ON NUCLEIC ACID PHOTOCHEMISTRY

Thesis by

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ABSTRACT

I. Frozen aqueous solutions of thymine and N,N'-dimethylthymine have been irradiated at 2537 Å to yield two dimers from N,N'-dimethylthymine and a single dimer from thymine. In all cases irradiation of the dimer in liquid aqueous solution at 2537 Å causes reversion to the monomer. One of the photodimers obtained from N,N'-dimethylthymine is identical to a product obtained by exhaustively N-methylating the single photodimer obtained from thymine. Nuclear magnetic resonance and ultraviolet absorption data support a structure containing a cyclobutane ring.

II. Action spectra for formation of thymine dimer in E. coli DNA have been taken. The initial quantum yield is not strongly dependent on wavelength. The ratio of thymine dimer to thymine in the photostationary state is much more dependent on wavelength. At the 235 mμ photosteady state 1.7% of the thymine is present as dimer. This shifts to 6.5% at 254 mμ and to 20% at 275 mμ. While the change in the position of the photosteady state with wavelength fails to fit a simple model, the data do indicate that not all thymines are capable of participating in dimer formation.

III. Irradiation of ultraviolet irradiated DNA with photoreactivating light (370 mμ) in the presence of an extract from baker's yeast containing photoreactivating enzyme causes the disappearance of thymine photodimer. The agent causing thymine photodimer to disappear is heat labile. These results suggest that the molecular basis of photoreactivation of biological damage to microorganisms is the reconversion of thymine photodimer to thymine.

TABLE OF CONTENTS

I. INTRODUCTION

Ever greater attention is being focused on nucleic acid photochemistry, not only because of the discovery of some rather novel photochemical reactions, but also because of the implication of nucleic acids as the primary site of ultraviolet damage to microorganisms. The goal of this type of research is first of all to find out what photochemical changes take place in nucleic acids and then to measure the quantum yields for these reactions. Finally one would like to know which photochemical damages account for the biological effects of ultraviolet light upon microorganisms.

In the work reported in this thesis, information pertaining both to nucleic acid photochemistry and ultraviolet radiobiology has been obtained about a particular ultraviolet event, the conversion of thymine to thymine photodimer.

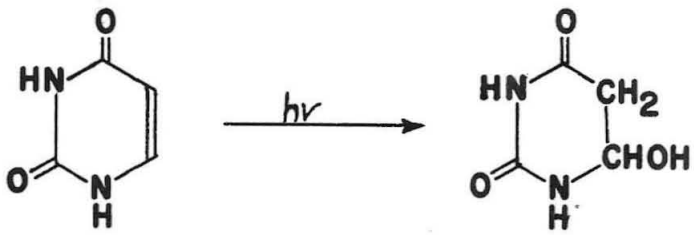
II. A REVIEW OF NUCLEIC ACID PHOTOCHEMISTRY

In 1958 there appeared in the Dutch chemical journal Recueil des Travaux Chimiques des Pays-Bas the first of a series of papers by Beukers, Berends and coworkers (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) which were destined to stimulate a rapid increase in our understanding of nucleic acid photochemistry. Indeed, the advances initiated by their discovery of thymine photodimer are so profound that the very excellent review article recently written by Shugar (11) is all but antiquated. Therefore I have sought to review the status of the field in the light of these recent discoveries and to place the role of thymine dimerization in nucleic acid photochemistry in perspective with the many other photochemical events which take place in DNA. The review is not intended to be comprehensive; it is intended to tell the important aspects of the story.

Photochemical alterations of nucleic acid components

General features. It was reported some time ago that pyrimidines are generally more sensitive to ultraviolet irradiation than purines (12, 13). Few exceptions to this observation have been reported and all four pyrimidines which occur commonly in nucleic acids, thymine, uracil, cytosine, and (in T-even bacteriophages) 5-hydroxymethylcytosine, are more sensitive to ultraviolet light than the commonly occurring purines, adenine and guanine (11).

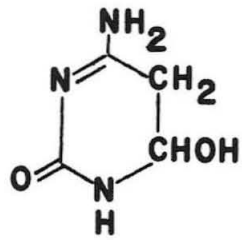
Hydration. It was not until 1949, when Sinsheimer found that uracil photochemically added water across its 5,6-double bond (14, 15), that a "one-hit" photochemical reaction was characterized for a nucleic acid component:



Although the photoproduct is stable at room temperature, either heat, acid or alkali will catalyze its reconversion to uracil. The -OH group has been shown to reside at the 6-position on the uracil ring (16, 17, 18, 19) and other nucleophiles, such as $-OCH_3$ and -CN, will add instead of -OH under appropriate conditions (20, 21, 22, 23).

Thymine will not form a stable water adduct upon ultraviolet irradiation although Wang has hypothesized that it forms a transient water adduct (24). Very little is known about 5-hydroxymethylcytosine photochemistry (25).

Cytosine seems to undergo a reaction similar to uracil, but its photoproduct is rather unstable and reverts back to cytosine in a matter of hours, even at 0°C (26). A controversy is currently raging over whether the photoproduct of cytosine is really a water adduct (27, 28) or merely involves a proton shift (29):



I

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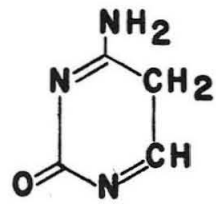

II

Dr. Hammond has pointed out to me that if the primary photochemical product is the water adduct (I) in analogy with uracil, it is not at all unreasonable that it should rapidly revert to compound II , giving a net reaction of a proton shift (30). Work in progress in Dr. Wang's laboratory (31) should shortly resolve this question.

Dimerization in frozen solution. An important advance in our understanding of nucleic acid photochemistry was made by Beukers and Berends, who irradiated frozen aqueous solutions of nucleic acid components (2). They found that frozen aqueous solutions of thymine derivatives were much more sensitive to ultraviolet irradiation, as evidenced by loss of the 264 mμ absorption band, than the corresponding thawed solutions. Moreover, they made the exciting discovery that when a frozen solution was irradiated and then thawed, irradiation with 254 mμ ultraviolet light caused the 264 mμ absorption band of thymine to reappear (5). Over a period of a year or so the nature of this phenomenon became clear. The product of ultraviolet irradiation of frozen aqueous solutions of thymine is a dimer, bonded across the 5,6-double bonds of two thymines (8, 9, 23, 32). Both monomer → dimer and

dimer → monomer reactions are photochemically induced. However, the rate of the forward reaction will depend additionally upon the proximity of the 5,6-double bonds of the two thymine molecules which are to dimerize. With photolysis of quite dilute thymine solutions ($\sim 10^{-4}$ M), customarily used for study in years past because of their convenience for spectrophotometric assay, the photosteady state lies almost entirely on the side of the monomer (1). Upon freezing, thymine molecules are postulated to become concentrated into microaggregates in which the 5,6-double bonds of two adjacent thymines happen to be favorably oriented for dimer formation (23).

Observations which support this interpretation include: (1) Irradiation of concentrated thymidine solutions (~ 1 M) with 254 mμ ultraviolet light causes a decrease of absorption at 264 mμ. Upon diluting the solution, further ultraviolet irradiation causes an increase in absorption at 264 mμ (33). (2) That something more than mere concentration of thymine molecules is required is supported by the observation by Mr. R. F. Stewart that thin crystals of 1-methyl thymine are more sensitive to ultraviolet irradiation than the Hoogsteen adenine-thymine hydrogen bonded dimer (34). Although crystals of both compounds contain thymine molecules stacked upon one another, the 5,6-double bonds of adjacent thymines in 1-methyl thymine are in direct contact with one another, while in the case of the Hoogsteen dimer they are not (35,36). (3) Wang irradiated thin films of thymine which had been formed by slow

evaporation of aqueous thymine solutions. Sometimes he obtained thymine dimer; sometimes no reaction occurred (23, 37). This result may be rationalized by saying that the thymine photodimer obtained in this way originates from thymine monohydrate, which has a crystal structure such that the 5, 6-double bonds of adjacent thymines are favorably oriented for photodimer formation (38). Thymine monohydrate crystals are unstable at humidities less than 70% (38). If one assumes that the dehydration product of thymine monohydrate does not have close 5, 6-double bonds, a plausible explanation for Wang's frequent failure to obtain dimer by this evaporation technique is at once found. My failure to obtain thymine photodimer by ultraviolet irradiation of thin crystals of anhydrous thymine (crystal structure unknown) may be rationalized in a similar manner (39).

Among other nucleic acid components only uracil has been shown to dimerize upon ultraviolet irradiation of its frozen aqueous solution (2, 23, 40, 41, 42, 43). Many N-substituted thymines and uracils undergo similar frozen solution photochemistry (2, 23, 32, 43, 44). In general with these compounds, complete conversion to dimer cannot be obtained, which presumably means that the monomer $\rightleftharpoons$ dimer photosteady states of these compounds lie more on the side of monomer than does the photosteady state characteristic of frozen thymine solutions.

Thymine photodimer is not formed upon ultraviolet irradiation

of frozen aqueous solutions of thymine containing 5% glycerol or methanol (5). If an aqueous solution of thymine photodimer containing 5% glycerol or methanol is frozen and irradiated, thymine is obtained (9). These results indicate that the photosteady state is apparently shifted to the side of the monomer by addition of methanol or glycerol before freezing. The reason for this shift is suggested by the results of Wang (23), who irradiated a frozen aqueous solution of uracil with 5% methanol added. He obtained addition of methanol across the 5,6-double bond of uracil, in the same manner as water would have added upon irradiation of the thawed solution. Wang has interpreted this to mean that freezing of uracil in methanolic water causes "puddles" of uracil in methanol to be formed instead of the usual microcrystals of uracil. Addition of methanol across the 5,6-double bond would then take place upon ultraviolet irradiation.

In addition to organic solvents such as methanol and glycerol, adenine and 5-bromouracil (5-BU) inhibit thymine photodimer formation, although cytosine has no effect (45). Mixtures of uracil and thymine yield uracil-thymine hybrids in addition to the homologous uracil and thymine dimers (41,42). Although 5-BU has no increased photosensitivity upon irradiation in frozen solution, mixtures of 5-BU and cytosine-2-C-14 yield several photoproducts, all of which are not radioactive (45). Mixtures of uracil and 5-BU give photoproducts derived only from uracil, only from 5-BU and 5-BU-uracil crossproducts (45).

Apparently the intermolecular orientation which occurs in a frozen solution of a single purine or pyrimidine may be sometimes altered by freezing in the presence of another purine or pyrimidine.

Dimerization: The effect of paramagnetic ions. The photosteady state of orotic acid in dilute (fluid) aqueous solution is such that an appreciable proportion of the orotic acid is dimerized, even at concentrations as low as 10^{-4} M (4). Addition of oxygen or paramagnetic ions to the solution pushes the photosteady state to the side of the monomer; removal of oxygen and paramagnetic ions shifts it towards the dimer (4, 7). A study of the effect of paramagnetic ions on the individual forward and back reactions has yet to be done, as has an investigation of a possible paramagnetic ion effect on photodimerization in concentrated thymidine solutions. It is tempting to speculate that paramagnetic ions act by retarding the forward reaction by quenching a triplet state intermediate.

Action spectrum of dimerization in a dinucleotide. Since thymine photodimerization is favored by bringing the 5, 6-double bonds of adjacent thymines close to each other, one might expect that irradiation of a dilute solution of thymine dinucleotide would result in appreciable thymine photodimerization. Indeed irradiation of thymine dinucleotide with 254 mμ ultraviolet light brings about a photosteady state in which approximately 30% of the thymines exist as photodimer (9, 42, 44).

The position of the photosteady state in thymine dinucleotide is

strikingly dependent upon the wavelength of light used: Almost all thymines become photodimerized at the photosteady state upon irradiation with 275 mμ light, while the photosteady state lies almost entirely on the side of the monomer upon irradiation with 235 mμ light (46). The quantum yield of both the forward and back reactions is relatively little affected by wavelength, but the ratio of the extinction coefficient of the dimer to that of the monomer varies by a factor of about 200 between 275 mμ and 235 mμ (46, 47), thus causing the dramatic wavelength dependence observed for the position of the photosteady state.

Photochemistry of DNA

Hydration. It is extremely difficult to say what role hydration has in the photochemistry of DNA. Spectral studies of photohydration in nucleic acids (48, 49, 50) are difficult to interpret because one does not know how any given photochemical lesion will affect the extinction coefficients of nearby undamaged nucleotides. Also one does not know what part of the observed spectral changes are due to photohydration and what part are due to competing photochemical reactions. The instability of cytosine photohydrate would make it impossible to estimate the concentration of this component by direct hydrolysis of irradiated DNA.

The stability of cytosine photohydrate in irradiated DNA is not known. It has been speculated that thymine in DNA will form a stable photohydrate even though it forms no stable photohydrate by itself (24).

Dimerization. Thymine dimer has been isolated from acid hydrolysates of DNA (6, 51). A photosteady state with about 10% of the thymine converted to thymine dimer is formed by sufficient irradiation of E. coli DNA with 254 mμ ultraviolet light (42). In work reported in this thesis, it is shown that the dependence of the position of the photosteady state on wavelength of light used for irradiation is roughly the same as was observed by Johns et al. for thymine dinucleotide (46), except that an appreciable fraction of the thymines do not seem to be able to partake in dimerization. The results are roughly consistent with the idea that only thymine-thymine nearest neighbor pairs are able to dimerize.

There has been some speculation that thymine photodimer formation in DNA is preceded by a "softening up" of the DNA by other photochemical damages. In such a case one would find that no thymine dimer is formed below a certain dose. Thymine dimer formation is linear with dose down to 3000 ergs/mm^2 at 254 mμ; reliable data at lower doses have not yet been published.

It is not known whether cytosine-cytosine photodimers or cytosine-thymine photohybrids are formed in ultraviolet irradiated DNA.

Physical properties. Several workers have studied the effect of ultraviolet light on the physical properties of DNA (48, 52, 53, 54). It seems clear that little or no detectable changes in most physical properties occur at ultraviolet doses below those needed to establish the thymine-

thymine photodimer steady state at 254 mμ. However, at sufficiently high ultraviolet doses several changes characteristic of a general degradation and decrease in stability of the DNA structure occur, including an oxygen accelerated decrease in viscosity and molecular weight and an increase in sensitivity to heat, acid, and formaldehyde denaturation. With somewhat lower ultraviolet dose, an increase in the density at which the DNA bands in a cesium chloride density gradient occurs (52, 53, 54).

Crosslinking. Irradiation of a dry film of DNA results in the formation of crosslinks between molecules (55, 56).

A certain amount of crosslinking also occurs in ultraviolet irradiated aqueous solutions of DNA (52, 53, 54). The crosslinks seem to be formed only between two strands of the same DNA molecule. It has been hypothesized that these crosslinks are due to thymine dimerization. At a dose when about half of the DNA molecules are crosslinked, there are about 100 thymine dimers per DNA molecule. Thus, if the crosslinking is due to thymine dimerization, it represents only a small fraction of the total thymine dimer present in the system. It is not known whether crosslinks can form at very low doses; it is quite conceivable that they should form only after primary damages "soften up" the DNA sufficiently.

The molecular basis of ultraviolet death to microorganisms

The preponderant biological effect of ultraviolet light on

microorganisms is a killing action. This killing seems to be intimately associated with some sort of damage to the DNA of the microorganism; the molecular nature of the ultraviolet lesions is not known (57).

It has been generally speculated that thymine dimer is this lethal lesion ever since its discovery by Beukers and Berends. Perhaps the simplest and best suited system from a biological point of view for investigating the validity of this hypothesis is the ultraviolet inactivation of the T-even bacteriophages. At present no direct comparison of the number of thymine dimers per bacteriophage per lethal hit has been published. It is not only necessary to measure the ultraviolet cross-section for thymine dimer formation in the bacteriophages themselves, but it is also necessary to measure the cross-section for dimer formation at the extremely low doses needed to kill bacteriophages because of the possibility that dimer formation takes place only after a primary ultraviolet lesion first "softens up" the DNA.

The role of other possible damages in ultraviolet inactivation is in general not understood. However, there is some indication that if any heat reversible damages (such as cytosine hydration) are produced upon ultraviolet irradiation of biologically active transforming DNA, they do not cause loss of biological activity (53).

Thymine photodimer and photoreactivation

"Photoreactivation" is a term used to describe a phenomenon whereby the survival probability of an ultraviolet irradiated microorganism

is found, in some cases and under some circumstances, to be markedly higher if the organism is incubated in the presence of 370 mμ light following ultraviolet irradiation. (See Jagger (58) for an excellent review article on this subject.) Rupert has obtained an enzyme preparation from baker's yeast which can photoreactivate ultraviolet damage to biologically active transforming DNA (59, 60, 61). This enzyme forms a complex specifically with ultraviolet irradiated DNA. Upon incubation in the presence of photoreactivating light, photoreactivable damages are repaired and the enzyme-substrate complex dissociates. In this thesis it is shown that, in the presence of the enzyme preparation, irradiation with photoreactivating light (370 mμ) of u.v. damaged DNA causes thymine dimer to disappear. These results strongly suggest that thymine dimer is an important cause of ultraviolet inactivation of transforming DNA and that photoreactivating enzyme restores biological activity to transforming DNA by reconverting thymine dimer to thymine.

ON THE NATURE OF THYMINE PHOTOPRODUCT*

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SUMMARY

Frozen aqueous solutions of thymine and N,N'-dimethylthymine have been irradiated at 2537 Å to yield two dimers from N,N'-dimethylthymine and a single dimer from thymine. In all cases irradiation of the dimer in liquid aqueous solution at 2537 Å causes reversion to the monomer. One of the photodimers obtained from N,N'-dimethylthymine is identical to a product obtained by exhaustively N-methylating the single photodimer obtained from thymine. Nuclear magnetic resonance and ultraviolet absorption data support a structure containing a cyclobutane ring. Four different isomers consistent with these data are depicted in the text.

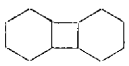
Chemical shifts and spin coupling constants have been obtained for thymine and the three dimers.

INTRODUCTION

BEUKERS, IJLSTRA AND BERENDS[1] have found that thymine in frozen aqueous solution is converted by ultraviolet light into a new compound which has no thymine-like ultraviolet absorption band near 265 mμ. This photoproduct, which may be iso-

* Contribution No. 2654.

lated in crystalline form, is stable in 6 *N* NaOH and fuming perchloric acid. BEUKERS *et al.*[2] made the interesting and exciting discovery that ultraviolet irradiation of a liquid aqueous solution of thymine photoproduct brings about the restoration of thymine. BEUKERS AND BERENDS have shown that thymine photoproduct has the same empirical formula as thymine and a molecular weight twice that of thymine[3]. Infrared absorption bands are found for thymine photoproduct in the neighborhood of 870 cm^{-1}. BEUKERS AND BERENDS have attributed these bands to cyclobutane ring vibrations. This evidence and the "improbability of other possibilities" have led them to the conclusion that thymine photoproduct has the following structural skeleton:



Formation of the cyclobutane ring is accompanied by saturation of the 5,6 double bond of thymine. BEUKERS AND BERENDS state further that nuclear magnetic resonance spectra show no spin–spin splitting of the proton attached to the 6 carbon atom of one thymine ring by the proton attached to the 6 carbon atom of the other thymine ring and that the isomer formed must therefore be one in which the 6 carbon atoms are not adjacent. This argument is invalid since these hydrogens are in equivalent magnetic environments, regardless of isomer; hence in this case no spin–spin splitting is to be expected[4].

Not only is the photochemically reversible conversion of thymine into a dimer interesting as a purely chemical phenomenon, but such alteration of thymine may be the primary source of ultraviolet damage to the genetic function of microorganisms. Indeed thymine photoproduct has been isolated from irradiated aqueous solutions of deoxyribonucleic acid[5] and from irradiated bacteria[6]. The question as to whether or not the photochemical reconversion of thymine photoproduct to thymine is related to the biological phenomenon of photoreactivation is one which invites further investigation.

In this communication we wish to report structural investigations of compounds produced by ultraviolet irradiation of frozen solutions of thymine and N,N'-dimethylthymine.

MATERIALS AND METHODS

General

All materials were of reagent grade unless noted otherwise. Thymine of CfP grade was obtained from the California Corporation for Biochemical Research. Dimethyl sulfoxide, from the Crown Zellerbach Corporation, was dried with Davison molecular sieve powder and fractionated at 3.5 mm, b.p. 39.5–40.5°. Dimethyl-d_6 sulfoxide (99 %D) was obtained from Merck and Co., Ltd. of Canada. Deuterium oxide (99 %D) was obtained from General Dynamics Corporation.

Molecular weights were determined to $\pm$ 10 % by freezing point depression in camphor.

Ultraviolet absorption measurements were made either with a Beckman Model DU or with a Cary Model 14 spectrophotometer.

Nuclear magnetic resonance spectra were determined with the Varian high resolution nuclear magnetic resonance spectrometer, using a R.F. unit for 60 Mc, as

described previously[7]. Chemical shifts were calibrated by the method of side-band audiomodulation with an accuracy of ± 1 cyc./sec (see ref. 8). The solvent peaks (dimethyl sulfoxide and water) were located relative to hexane as an external standard in separate experiments and the solute peaks were determined relative to the solvent peaks. The proton resonance in dimethyl sulfoxide occurs at +7.50 on the Tiers scale. Solutions used for nuclear magnetic resonance analysis were approx. 5 %.

Preparation of thymine photoproduct

A 100-ml portion of a 1 % thymine solution was cooled to −78° in a shallow aluminum refrigerator tray of surface area 600 cm². The frozen solution was irradiated for about 1 h with two 8-W General Electric germicidal lamps placed about 5 cm from the surface of the solution. About 60 % conversion to photoproduct was obtained in this way. Several batches of thawed solution were concentrated to one-fourth of the original volume, using a steam bath and water aspirator. The solution was heated to 100°, filtered to remove debris, and allowed to crystallize overnight at 4°. Thymine photoproduct (solubility 0.5 g/l in water at room temperature) precipitated while thymine (solubility 4 g/l in water at room temperature) stayed in solution. The crystalline precipitate was recrystallized from hot water and oven-dried at 100°. Long white needles were obtained. Since no trace of an aromatic absorption band centered at 265 mμ could be detected spectrophotometrically (Fig. 1), it was concluded that no thymine was present in this material.

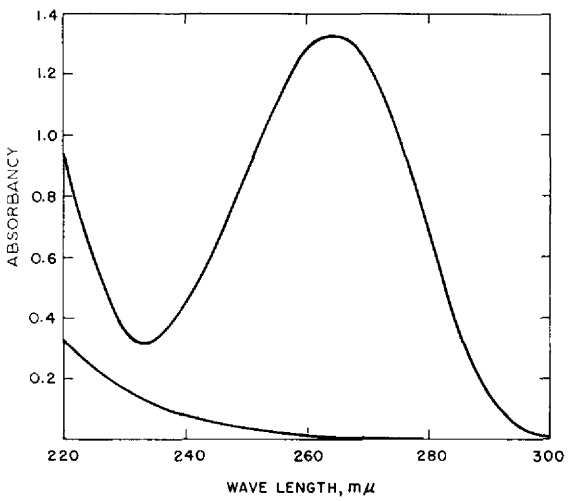


Fig. 1. Upper line is the absorbancy of a 20 μg/ml ($1.6 \cdot 10^{-4}$ *M*) solution of thymine; lower line is the absorbancy of a 20 μg/ml solution of thymine photoproduct.

Methylation of thymine photoproduct

Sodium hydroxide (0.5 g) was dissolved in 6 ml of water and thymine photoproduct (0.7 g) was added. The resulting slurry was cooled in an ice bath and 1.4 ml of dimethyl sulfate was added dropwise over a period of about 5 min with continuous stirring. Stirring was continued for about 4 h at 0° and then overnight at room temperature. The solution was extracted with three 10-ml portions of chloroform. The chloro-

form extracts were evaporated and there remained a viscous brown oil. Most of the oil dissolved upon addition of 20 ml of hot benzene; the residue was discarded. An alumina column of dimensions 1.8 cm^2 × 10 cm was prepared from a slurry of alumina ("acid washed" from Merck and Co., Inc.) in hexane. 10 ml of benzene were passed through the column, followed by the solution of methylated photoproduct in benzene. Elution was carried out by adding solvent to the column as follows: 50 ml of benzene, 50 ml of benzene–ether (1:1), 50 ml of ether, 50 ml of 1 % ethanol in ether, 50 ml of 2 % ethanol in ether, etc. up to 50 ml of 12 % ethanol in ether, and then finally 100 ml of ethanol. 20-ml aliquots were collected and evaporated to dryness. A crystalline material was eluted in 9–10 % ethanol yielding, upon recrystallization from chloroform–hexane, white crystals in the form of hexagonal right prisms, m.p. 246–247°.

Anal. Calcd. for $C_7H_{10}N_2O_2$: C, 54.53 %; H, 6.54 %; N, 18.17 %. Found: C, 54.63 %; H, 6.47 %; N, 18.35 %. Mol. wt. for $(C_7H_{10}N_2O_2)_2$: 308. Found: 321.

Irradiation of N,N'-dimethylthymine

N,N'-Dimethylthymine was prepared from thymine by the method of DAVIDSON AND BAUDISH[9]. A 2.5 % aqueous solution was irradiated in the same manner as was thymine. However, dimethylthymine could only be irradiated to about 60 % loss of absorbancy at its maximum of 270 mμ; further loss of absorbancy at 270 mμ could not be effected by thawing the solution, refreezing, and then irradiating again. The irradiated solution was evaporated on a steam bath under reduced pressure to give a yellow oil. Final traces of moisture were removed by drying *in vacuo* over anhydrous calcium sulfate. One gram of residue was placed on top of a freshly prepared alumina column of dimensions 1.8 cm^2 × 19 cm (in retrospect, we think it would be better to first dissolve the powder in hot benzene) and eluted with benzene, benzene–ether, ether, ether–ethanol, and ethanol. Aliquots were evaporated to dryness and residues were recrystallized from chloroform–hexane. A crystalline material which was eluted with benzene–ether had an ultraviolet absorption spectrum identical to that of N,N'-dimethylthymine; while a crystalline material which was eluted from ether–ethanol had an ultraviolet absorption spectrum similar to that of thymine photoproduct. Since nuclear magnetic resonance spectra indicated that the ether–ethanol eluate was a mixture of two photoproducts, further separation on alumina was carried out: The recrystallized ether–ethanol eluate was placed as a powder upon a freshly prepared alumina column (1.8 cm^2 × 19 cm) and eluted with successive 100-ml portions of diethyl ether, 1 % ethanol in ether, 2 % ethanol in ether etc., to 12 % ethanol in ether, and then finally with ethanol. 10-ml fractions were collected and evaporated to dryness. Residue first appeared upon elution of the column with 4–5 % ethanol, disappeared upon elution with 6–7 % ethanol, and then reappeared upon elution with 8–9 % ethanol. The residue eluted by 4–5 % ethanol was combined and recrystallized from chloroform–hexane to give white crystals in the form of parallelepipeds, m.p. 224–225°, and was labelled dimethylthymine photoproduct I. The residue from 8–9 % ethanol was treated similarly to give white crystals in the form of hexagonal right prisms, m.p. 250–251°, and was labelled dimethylthymine photoproduct II. The nuclear magnetic resonance spectra of dimethylthymine photoproducts I and II, which are formed in approximately equal amounts, can be superimposed to give the nuclear magnetic resonance spectra of the material before separation.

Anal. Calcd. for $C_7H_{12}N_2O_2$: C, 54.53%; H, 6.54%; N, 18.17%. Found for dimethylthymine photoproduct I: C, 54.51%; H, 6.57%; N, 18.17%. Found for dimethylthymine photoproduct II: C, 54.35%; H, 6.50%; N, 18.16%. Mol. wt. for $(C_7H_{12}N_2O_2)_2$: 308. Found for N,N'-dimethylthymine photoproduct I: 292. Found for N,N'-dimethylthymine photoproduct II: 289.

RESULTS AND DISCUSSION

Nuclear magnetic resonance spectra of thymine

Since both thymine and thymine photoproduct are quite insoluble in water, it was necessary to use either perchloric acid or dimethyl sulfoxide as a solvent for nuclear magnetic resonance studies. Both solvents were used and yielded similar results, although better resolution was obtained in dimethyl sulfoxide.

Fig. 2a shows the nuclear magnetic resonance spectrum of thymine in dimethyl sulfoxide. The peaks due to the two hydrogens that are bonded to nitrogen (referred to as "NH" peaks) are identified by their appearance at extremely low fields and by their disappearance upon addition of a small amount of D_2O to the solution. The broader NH peak is due to the NH adjacent to CH while the other is derived from CONHCO. Of the two peaks derived from protons that will not exchange with D_2O, one must be due to the hydrogen that is bonded to the 6 carbon atom of thymine (referred to as the "CH" peak) and the other must be due to the hydrogens of the 5 methyl group of thymine (referred to as the "C–CH_3" peak). These two peaks are identified on the basis of relative peak heights and chemical shifts. Further support of the assignment of absorption peaks as shown in Fig. 2a is given by an analysis of spin–spin splitting. While the two NH peaks are too broad to be resolved into subpeaks, the CH peak is split into two equal lines by the proton on the 1 nitrogen, $J_{CH,NH} = 5.7$ cyc./sec. This splitting disappears when the 1 nitrogen atom is deuter-

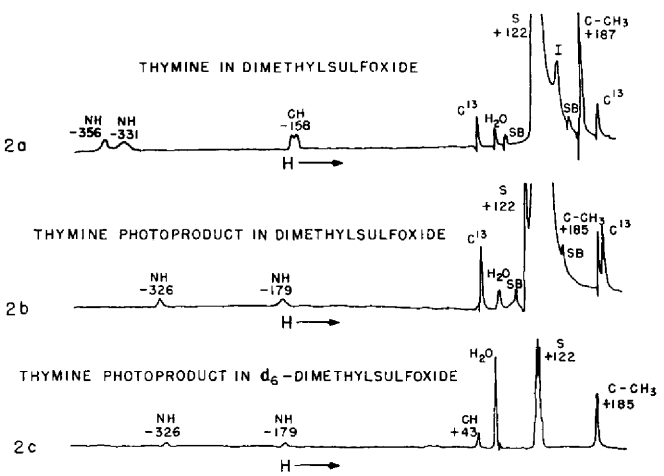


Fig. 2. Nuclear magnetic resonance spectra, 60 Mc, 29°. (a) Thymine in dimethyl sulfoxide. (b) Thymine photoproduct in dimethyl sulfoxide. (c) Thymine photoproduct in dimethyl-d_6 sulfoxide. Key: S, resonance of solvent protons; SB, spinning sideband from solvent; C^{13}, carbon thirteen satellite from solvent; H_2O, water or impurities in solvent that will rapidly exchange with water; I, impurity in solvent. The assignment of peaks is discussed in the text. In the figure the chemical shift is given in cyc./sec from external water. The magnetic field increases to the right.

ated, since $J_{H,D}/J_{H,H} \cong 1/7$, and $J_{CH,C-CH_3}$ is found to be 1.1 cyc./sec. The C–CH_3 peak is resolved into an equal doublet and the CH peak appears as a 1, 3, 3, 1 quartet. This last coupling constant is 1.1 cyc./sec in N,N′-dimethylthymine also.

Nuclear magnetic resonance spectra of thymine photoproduct

The nuclear magnetic resonance spectrum of thymine photoproduct in dimethyl sulfoxide is shown in Fig. 2b. Aside from absorption by the solvent, only three peaks are visible. Since it was thought that a fourth photoproduct peak might be hidden under a solvent peak, the nuclear magnetic resonance spectrum of thymine photoproduct was determined in dimethyl-d_6 sulfoxide. Indeed a fourth photoproduct peak appeared at the site of one of the ^{13}C satellite peaks of non-deuterated solvent, as shown in Fig. 2c.

The two NH peaks were established on the basis that they both disappeared upon addition of D_2O. The one furthest downfield disappeared in less than 5 min, while the other one disappeared over a period of 20 min. The fact that, even before addition of D_2O, they are smaller than the CH peak is attributed to exchange of nitrogen protons with deuterated exchangeable impurities in the dimethyl-d_6 sulfoxide.

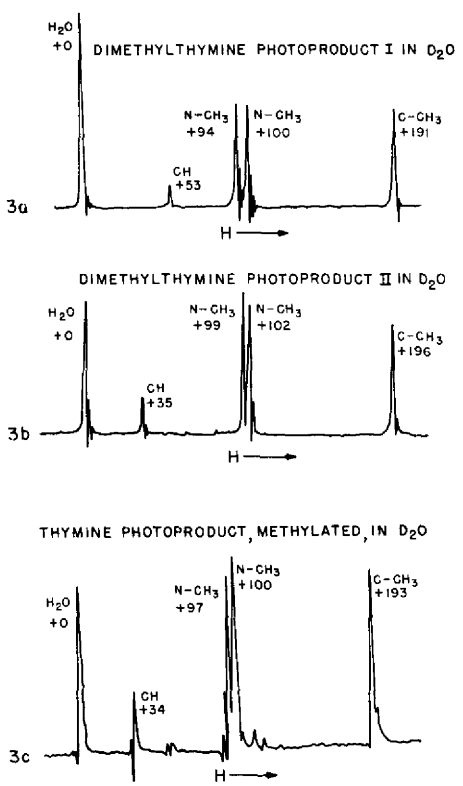


Fig. 3. Nuclear magnetic resonance spectra, 60 Mc, 29°. (a) N,N′-Dimethylthymine photoproduct I in D_2O. (b) N,N′-Dimethylthymine photoproduct II in D_2O. (c) Thymine photoproduct, methylated, in D_2O. The small unlabelled peaks in Fig. 3c are due to impurities. (A less pure sample than described in the experimental section was used for nuclear magnetic resonance studies.) Key: H_2O, resonance of solvent protons. The assignment of peaks is discussed in the text. In the figure the chemical shift is given in cyc./sec from the H_2O peak. The magnetic field increases to the right.

These peaks may be made to approach the CH peak height (in a solution to which no D_2O has been added) by making the solution about 1 % in H_2O. If no carbon-hydrogen bonds are broken in the photochemical process, which is quite reasonable on chemical grounds, the remaining two peaks must be the CH and the C–CH_3 peaks. They are assigned on the basis of relative peak heights, as shown in Fig. 2c.

Note the large chemical shift to higher field of the CH peak in thymine photoproduct as compared to the CH peak in thymine. Whereas the CH peak in thymine appears at a chemical shift typical for a proton on an aromatic ring, the CH peak in thymine photoproduct appears at a chemical shift typical for a proton on a saturated carbon atom. Thus nuclear magnetic resonance spectra point strongly to a saturated 5,6 double bond in thymine photoproduct.

The photoproducts of frozen N,N'-dimethylthymine solutions

Two photoproducts were isolated from irradiation of N,N'-dimethylthymine in frozen solutions as described above. These two compounds have the same empirical composition as N,N'-dimethylthymine and are dimeric; both lack an ultraviolet absorption-band characteristic of an aromatic ring. Both will revert to N,N'-dimethylthymine upon irradiation at 2537 Å in liquid aqueous solution as determined spectrophotometrically. The nuclear magnetic resonance spectra of these isomers in D_2O are shown in Fig. 3. With the exception of the N–CH_3 peaks, which have replaced the NH peaks, these spectra are quite similar to the nuclear magnetic resonance spectrum of thymine photoproduct in dimethyl-d_6 sulfoxide. In fact the chemical shift of the CH peaks strongly suggest that the 5,6 double bond has become saturated in these two dimethylthymine photodimers. All this evidence indicates that frozen dimethylthymine solutions may be irradiated to give two isomers, both of which have the structural skeleton proposed by BEUKERS AND BERENDS for thymine photoproduct.

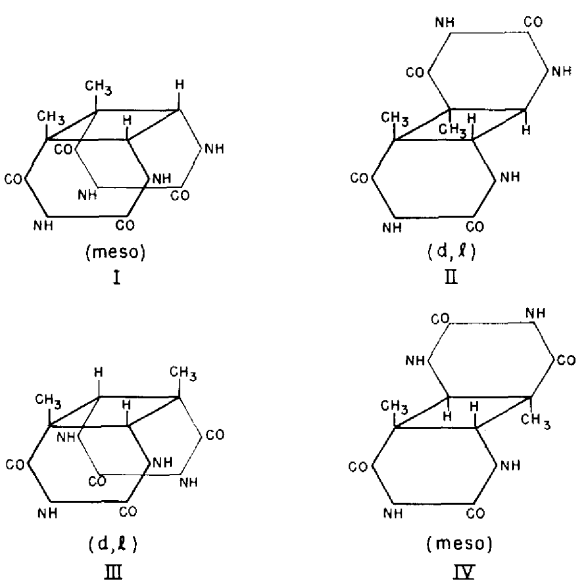


Fig 4. The four possible thymine dimers that contain a cyclobutane ring.

Comparison of thymine photoproduct with N,N'-dimethylthymine photoproducts I and II

In order to compare thymine photoproduct with the two photoproducts from N,N'-dimethylthymine, thymine photoproduct was first exhaustively N-methylated to give a compound with the empirical composition of dimethylthymine and a molecular weight twice that of dimethylthymine. The nuclear magnetic resonance spectrum of the methylated dimer (Fig. 3) and its elution behaviour on an alumina column indicated that it might be identical to N,N'-dimethylthymine photoproduct II. A mixed melting-point determination confirmed this hypothesis.

The four dimers with cyclobutane rings

There are four physically different dimers that have the empirical composition of thymine and that involve a cyclobutane ring formed by saturation of the 5,6 double bond (Fig. 4). It is impossible to tell from nuclear magnetic resonance studies alone which two of these isomers are obtained from N,N'-dimethylthymine.

Of these four isomers it is clear from inspection of molecular models that only I of Fig. 4 will arise from photodimerization of adjacent thymine moieties on deoxyribonucleic acid. On the other hand, cross linkage in irradiated DNA films[10] might arise from formation of one or both of the trans isomers, II and IV of Fig. 4. It is possible that all three of these isomers are formed *in vivo* under the proper conditions. This question requires further study.

ACKNOWLEDGEMENTS

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REFERENCES

1 R. BEUKERS, J. IJLSTRA AND W. BERENDS, *Rec. trav. chim.*, 77 (1958) 729.
2 R. BEUKERS, J. IJLSTRA AND W. BERENDS, *Rec. trav. chim.*, 78 (1959) 883.
3 R. BEUKERS AND W. BERENDS, *Biochim. Biophys. Acta*, 41 (1960) 550.
4 J. A. POPLE, W. G. SCHNEIDER AND H. J. BERNSTEIN, *High Resolution Nuclear Magnetic Resonance*, McGraw-Hill Book Company, Inc., New York, 1959, p. 87.
5 R. BEUKERS, J. IJLSTRA AND W. BERENDS, *Rec. trav. chim.*, 79 (1959) 101.
6 A. WACKER, H. DELLWEG AND D. WEINBLUM, *Naturwissenschaften*, 47 (1960) 477.
7 G. FRAENKEL AND C. FRANCONI, *J. Am. Chem. Soc.*, 82 (1960) 4478.
8 J. T. ARNOLD AND M. E. PACKARD, *J. Chem. Phys.*, 19 (1951) 1608.
9 D. DAVIDSON AND O. BAUDISH, *J. Am. Chem. Soc.*, 48 (1926) 2382.
10 D. SHUGAR AND G. BARANOWSKA, *Nature*, 185 (1960) 33.

IV. EXPERIMENTAL METHODS FOR WORK WITH DNA-THYMINE-H^3

Preparation of DNA-thymine-H^3

Radioactive isotope. Thymidine-methyl-H^3, 5 c/m$\underline{M}$ from New England Nuclear Corporation, was used without further purification.

Bacterial strain and growth conditions. The triple auxotroph E. coli 15 $T^-A^-U^-$ (requiring thymine, arginine, and uracil) was kindly supplied by Dr. P. Hanawalt and grown in a medium described by Maaløe and Hanawalt (62).

Incorporation of thymidine-methyl-H^3 into E. coli 15 $T^-A^-U^-$ DNA. An overnight culture of bacteria was harvested and resuspended at 5×10^7 per ml in medium containing 4 μg/μl of thymidine-methyl-H^3 (specific activity 1.3 c/m$\underline{M}$) as the source of thymine. The bacteria were allowed to grow to about 8×10^8 per ml and then collected by centrifugation.

Isolation and purification of DNA-thymine-H^3. The bacteria were washed with 1/40 volume saline-versene (0.15 $\underline{M}$ NaCl plus 0.1 $\underline{M}$ versene at pH 8), harvested by centrifugation and suspended in 0.7 ml of saline-versene (giving $\sim 10^{10}$ per ml). Lysis was effected by adding 0.05 ml of 25% dupanol and heating to 65°C for 10 minutes. Deproteinization was carried out by making the solution 1 $\underline{N}$ in $NaClO_4$ with 5 $\underline{N}$ $NaClO_4$, adding an equal volume of chloroform-isoamyl alcohol,

24:1 (v/v), and shaking for 15 minutes. After separation of the solution into two layers by centrifugation for 15 minutes at 5000 RPM in a Servall centrifuge, the aqueous phase was withdrawn and 1.35 g H_2O and 3.03 g of CsCl were added. The insoluble $CsClO_4$ was removed by a 5 minute centrifugation at 5000 RPM. After adjusting the density to $\rho = 1.70$ (determined by weighing 100 µl of solution), the solution was centrifuged in a lusteroid tube in a model L Spinco preparative centrifuge at 33,000 RPM at room temperature for 36 hours. After the rotor had coasted to a stop, the lusteroid tube was carefully secured and a pin hole was put in the bottom. Three drop fractions were collected in test tubes containing 0.6 ml of water. The fractions containing the DNA (tubes 13, 14 and 15 out of 27 tubes) were determined spectrophotometrically with a Cary Model 14 recording spectrophotometer and pooled. The concentration of radioactive label in the DNA fraction was about 20 times the concentration of radioactive label in tube 5. The DNA fraction was dialyzed at 4°C for 24 hours against two changes of 0.15 M NaCl, 0.015 M sodium citrate and stored at 4°C. (This procedure for preparing DNA is quite similar to that of Marmur (63).)

Ultraviolet irradiation of DNA

Irradiation of DNA solutions was performed using a General Electric BH6 high pressure mercury lamp and a quartz monochromater as described by Johns, Rappaport and Delbrück (46). The bandwidth between half peak intensity points was about 7 mµ. The intensity of the

lamp generally varied significantly with wavelength in the vicinity of the wavelength of irradiation. As a result, the effective wavelength of irradiation was up to 3 mμ different from the stated wavelength. The ultraviolet intensity was measured with a photocell which had been calibrated both with a standard thermopile and with the malachite green leucocyanide actinometer system (46).

Solutions containing 2 ml of 0.3 μg/ml of DNA (in 0.015 M NaCl, 0.0015 M sodium citrate) in a Beckman rectangular cell were stirred during irradiation with a glass stirring rod at 3350 RPM. For long irradiations, after about 10^5 ergs/mm^2 had been delivered, samples were stirred only intermittently because of evaporation and bubble formation. Samples for analysis were withdrawn from time to time in a 10 μl pipette. A correction was made for the loss in volume of the solution being irradiated, although this was small in comparison with experimental error.

Hydrolysis and chromatography of DNA

DNA samples were evaporated at 90°C with the aid of a gentle stream of air and subjected to formic acid hydrolysis (0.003 μg to 0.3 μg of DNA in 25 μl of 88% formic acid) in evacuated tubes at 175° for 30 minutes (64). The yield of thymine and thymine photodimer from irradiated DNA was independent of hydrolysis time over a range of 15 to 60 minutes at 175°C.

After hydrolysis, a sample was evaporated to dryness at room temperature with the aid of a gentle stream of air and the residue was taken up in 10 μl each of a 1 μg/μl solution of thymine photodimer in 6 N HCl and a 1 μg/μl solution of thymine. The sample was placed on the origin of an inch wide strip of Schleicher & Schuell 589 White Ribbon chromatography paper which had been previously washed by chromatography in ethyl acetate: acetic acid: water (10: 3: 1). The hydrolysis tube was washed with 1 drop of water, which was then also transferred to the paper. After drying, the sample was concentrated into a narrow band by a brief chromatography in water. (Both thymine and thymine photodimer move with the solvent front.)

The sample was then subjected to descending paper chromatography in isopropanol: conc. HCl: water (68: 15.5: 16.5).

In order to locate the position of thymine and thymine photodimer on the strip of paper, a strip of paper containing just carrier thymine and thymine dimer was chromatographed adjacent to each strip of paper containing radioactive sample. The thymine spot on this control paper was located by reason that thymine quenches fluorescence of ultraviolet irradiated paper. The thymine dimer spot on this control paper, after being reconverted to thymine by irradiation of the control paper strip for 8 minutes with a bank of four 16 watt germicidal lamps at 6 inches distance, could be detected by the ultraviolet fluorescence quenching method also. On the basis of this control, an intelligent guess was made as to the position of thymine photodimer in the paper containing tritiated

sample.

A strip of paper 4 cm long which supposedly contained thymine photodimer was cut (usually near R_F = 0.6) and clamped to the origin of another strip of washed paper with the aid of microscope slides and paper clips. The strip was subjected to chromatography in water until the solvent front had moved about 1 cm beyond the end of the microscope slides. After drying, the paper clips and microscope slides were removed and the strip was again subjected to a brief chromatography in water. This method served to quantitatively transfer thymine dimer from a diffuse spot on the original strip of paper into a narrow band on the new strip of paper.

This thymine dimer band was now subjected to descending chromatography in saturated ammonium sulfate: 1 N sodium acetate: isopropanol (40: 9: 1). Thymine dimer was located as before and a 4 cm strip (usually around R_F = 0.7) was cut as before.

Radioactive thymine from the first chromatography was located directly by the fluorescence quenching method and a 4 cm strip centered at the thymine spot was cut (usually around R_F = 0.8), as well as one 2 cm strip on each side.

All radioactive paper samples were transferred to the polyethylene vials used for liquid scintillation counting and 1 ml of water was added to each vial. The recovery of carrier thymine dimer from chromatography was now assayed spectrophotometrically in the following manner: After an overnight soaking, an aliquot of the thymine photodimer eluate was

placed into a 0.6 ml Beckman spectrophotometer cell. The ultraviolet spectrum was run on the Cary Model 14 before and after placing the sample for 10 minutes at 6 inches from a bank of four 16 watt germicidal lamps which caused maximal reconversion of carrier thymine dimer to thymine. The sample was returned to the vial after spectral analysis. This procedure was standardized by adding 1 ml of water containing 10 μg of thymine dimer to a 4 cm strip of paper which previously had been treated exactly as the 4 cm strip containing radioactive dimer. It was also necessary to do a control with blank paper and water because the absorption spectrum of the eluate of blank paper is depressed upon ultraviolet irradiation. (This correction generally amounted to about 15%.)

Regarding the accuracy of this method: (1) The spectrophotometric assay for recovery of carrier thymine dimer is reproducible to better than $\pm$ 10%. (2) No difference could be detected between the R_F value of thymine dimer prepared from frozen thymine solution and the R_F value for H^3-thymine dimer obtained from irradiated DNA (it is conceivable that these two dimers are isomers and not identical). (3) Each of the two 2 cm strips adjacent to the 4 cm thymine strip was less than 10% as radioactive as the thymine strip. All three strips together constituted 98% of the total radioactivity in unirradiated DNA and the sum of their activities was taken as the thymine activity for any given sample.

Estimation of thymine and thymine dimer by liquid scintillation counting

Tritium was counted in a Packard liquid scintillation spectrometer, using the dioxane-water (15 ml: 1 ml) system of Butler (65).

Concerning the accuracy in counting: (1) The paper from chromatography did not interfere with the counting and was left in the bottom of the sample vial. (2) The efficiency of counting was independent of the salts and impurities eluted from the paper into the system. (3) The radioactivity was quantitatively eluted upon overnight soaking. (4) The counts per minute of a sample remained the same for at least three months. (5) The counting of any given sample is accurate to $\pm$ 2% or better.

Treatment of DNA with an enzyme from baker's yeast

DNA which had been given 3100 ergs/mm^2 of 254 mμ ultraviolet light (2 ml of 0.7 μg/ml in 0.03 M NaCl, 0.003 M sodium citrate) was dialyzed 24 hours against 0.0015 M NaCl, 0.00015 M sodium citrate and concentrated to 200 μl at 60°C , using a gentle stream of nitrogen. The solution was adjusted to a volume of 280 μl and a salt concentration of 0.015 M NaCl, 0.0015 M sodium citrate. An aliquot was withdrawn for a direct determination of the fraction of thymine present as thymine dimer.

The partially purified (ammonium sulfate) preparation of yeast photoreactivating enzyme, sent to me by Dr. Rupert in the form of a filter cake, was dissolved in 0.01 M phosphate, pH 6.8, to give a total

concentration of 10 mg/ml and a protein concentration of 2500 μg/ml. (According to Dr. Rupert, the filter cake was 25% protein by weight.) One aliquot was heated at 65°C for 10 minutes. (Photoreactivating enzyme is reduced to 5% activity in 2 minutes at 65°C (60, 61).) Into each of two 1 dram vials containing 60 μl of DNA solution was placed 60 μl of unheated enzyme solution, into a third vial containing 60 μl of DNA solution was placed 60 μl of heated enzyme preparation. This gave a final concentration of 2.5 μg/μl DNA and 1250 μg/μl protein.

Photoreactivating light was supplied by "blacklight" fluorescent bulbs which transmit light between 300 and 400 mμ. The shorter wavelengths were filtered out with a sheet of window glass which had a cutoff wavelength of 340 mμ. The intensity of irradiation was calculated at about 2000 μwatts/cm^2 from the manufacturer's data.

Incubation mixtures were warmed to 37°C for 30 minutes either in the dark or in the light. On the basis of previous experience with transforming DNA, this would be expected to give maximum repair of the biologically significant damage (66). The mixtures were then diluted to 1 ml with 1.5 M NaCl and deproteinized by shaking with an equal volume of chloroform-octanol (9:1). The aqueous phase was dialyzed against 1.5×10^{-4} M NaCl, 1.5×10^{-5} M sodium citrate before analysis for thymine and thymine dimer.

About 70% of the radioactivity was routinely lost during this procedure.

V. EXPERIMENTAL RESULTS AND DISCUSSION: ACTION SPECTRA OF THYMINE DIMER FORMATION IN DNA

General features. Ultraviolet action spectra for the formation of thymine dimer in E. coli DNA are shown in figs. 1, 2 and 3. Qualitatively, the spectra may be interpreted to mean that thymine dimers are initially formed upon ultraviolet irradiation of DNA, but that at sufficiently high dose a photosteady state is formed between dimer and monomer. Evidence for a photoreversible system is shown in figs. 3, 4 and 5, where it is seen that the photosteady state characteristic of 275 mμ may be first converted to the 250 mμ photosteady state with 250 mμ light and then back to the 275 mμ photosteady state with 275 mμ light.

The photosteady state formed upon irradiation with 275 mμ light the second time lies more on the side of the monomer than the photosteady state initially formed by 275 mμ irradiation. One rationalization of this result is to say that the position of the photosteady state is affected by other ultraviolet damages to the DNA which slowly accumulate with increasing dose and are not reverted by 254 mμ light. Whatever the cause of the slow shift of the photosteady state towards monomer, it is clear that the dose required to bring about an appreciable shift in the photosteady state is somewhat larger than the dose required to form the photosteady state.

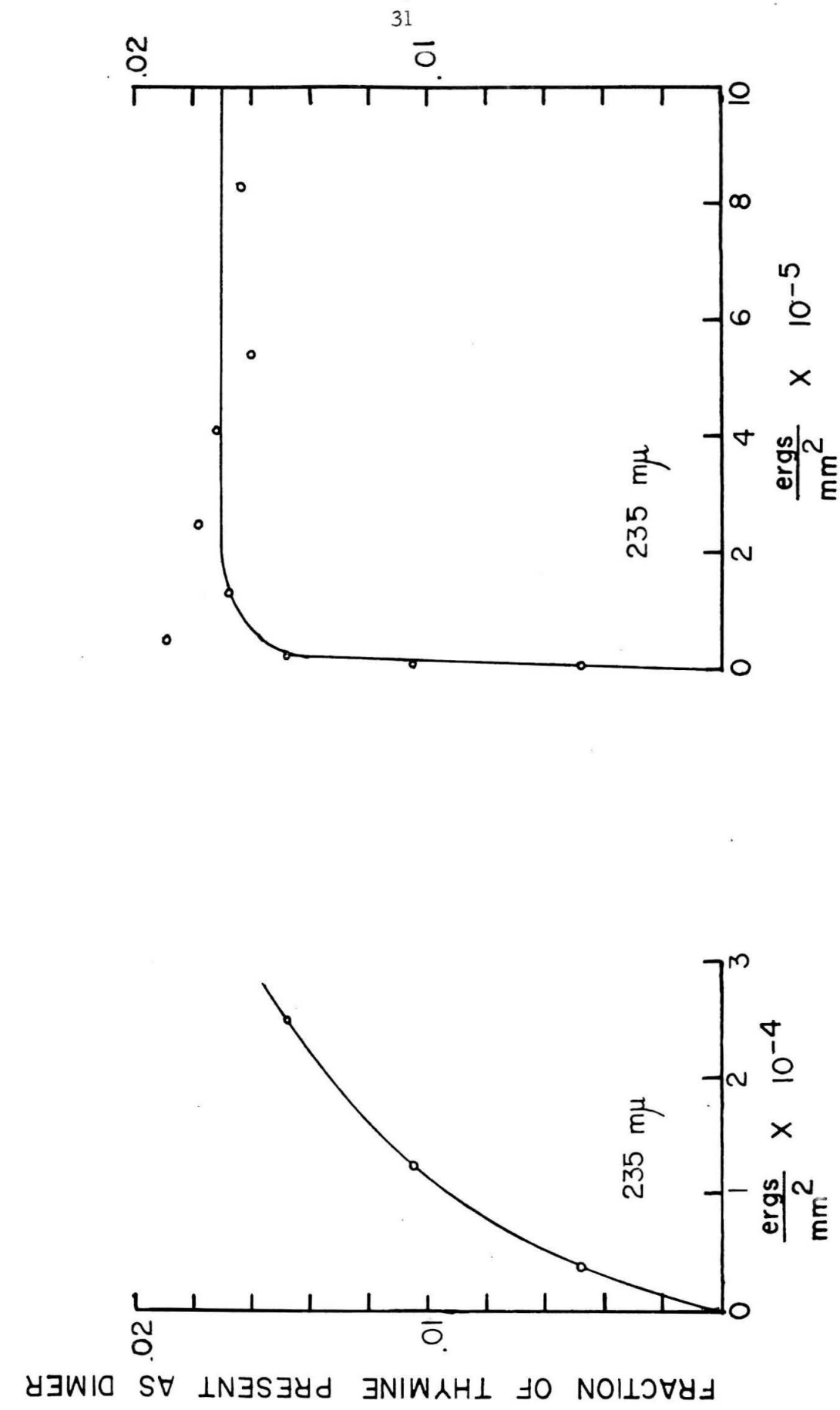

Fig. 1. Fraction of thymine present at dimer plotted against dose at 235 mμ.

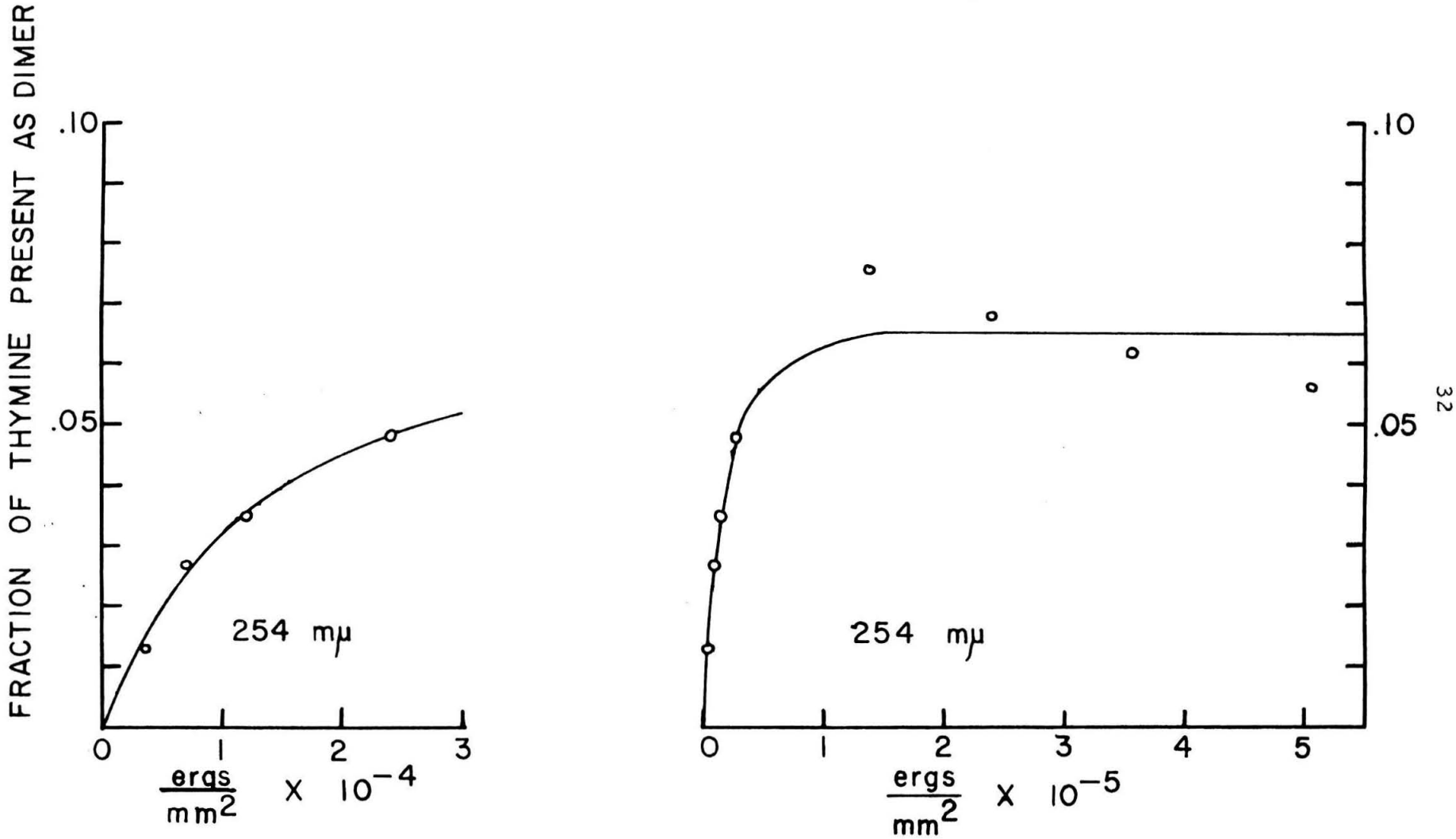

Fig. 2. Fraction of thymine present as dimer plotted against dose at 254 mμ.

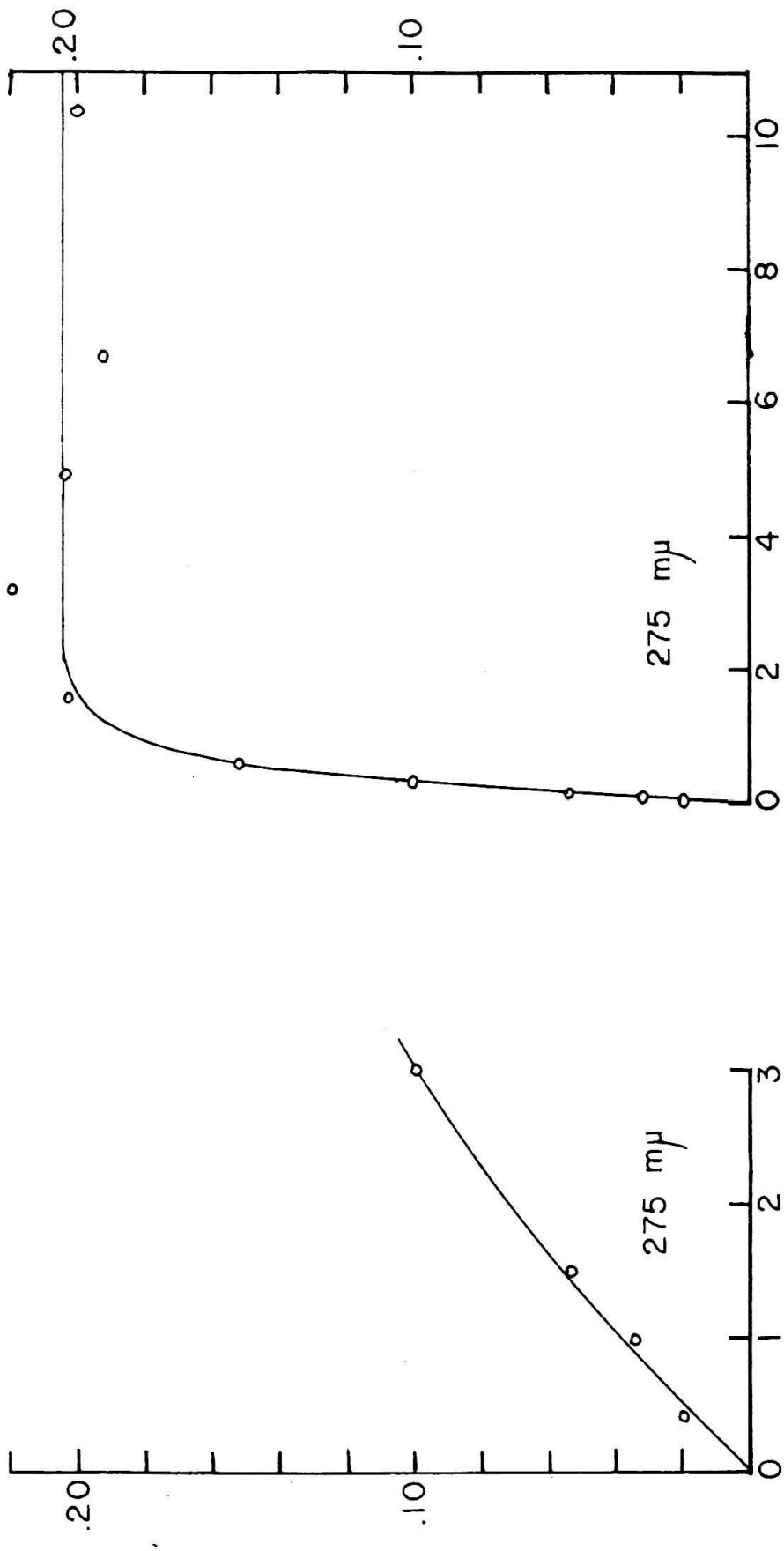

Fig. 3. Fraction of thymine present as dimer plotted against dose at 275 mμ.

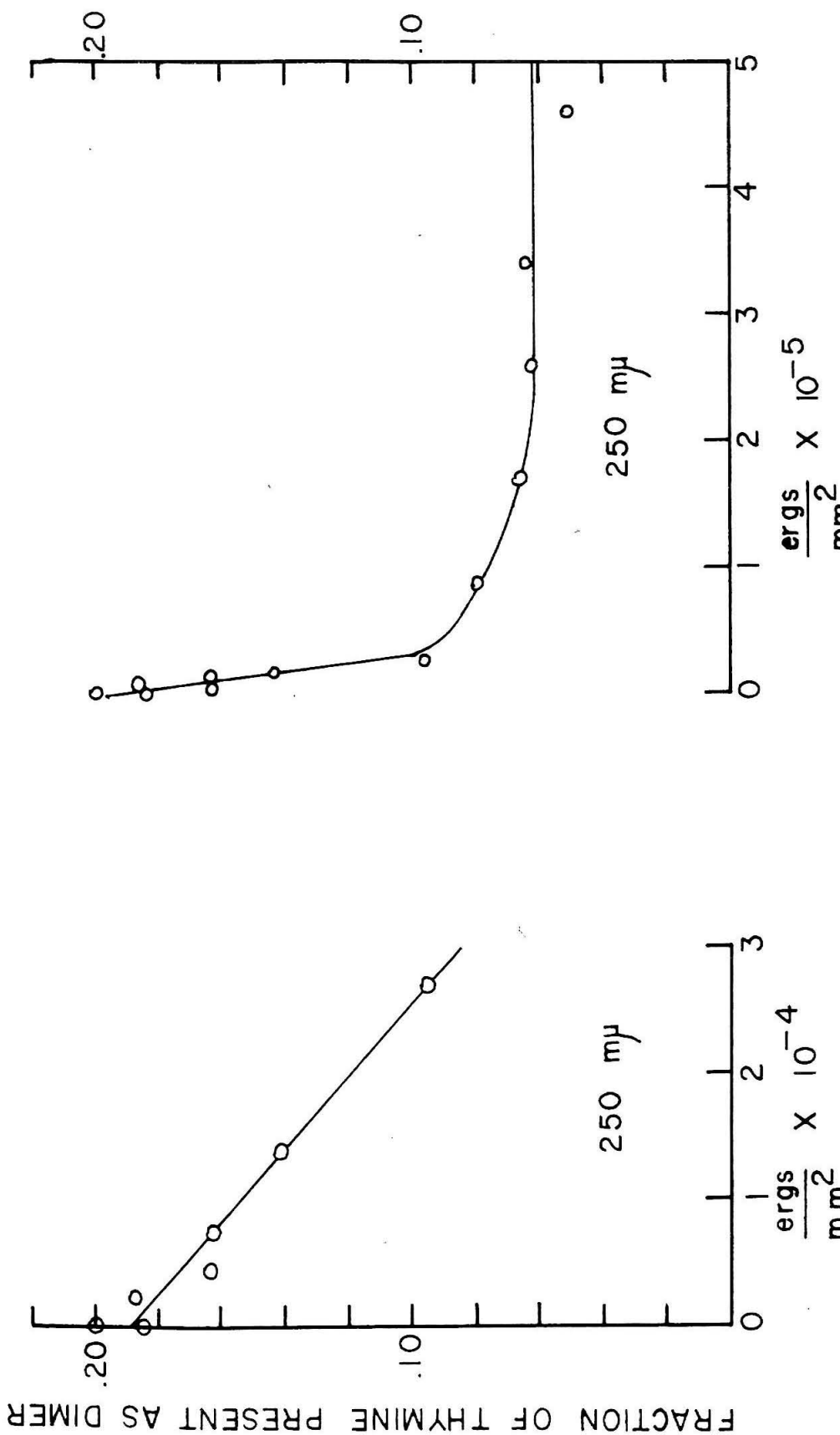

Fig. 4. Fraction of thymine present as dimer plotted against dose at 250 mμ for a sample which had previously been given 10^6 ergs/mm^2 of 275 mμ irradiation as shown in fig. 3.

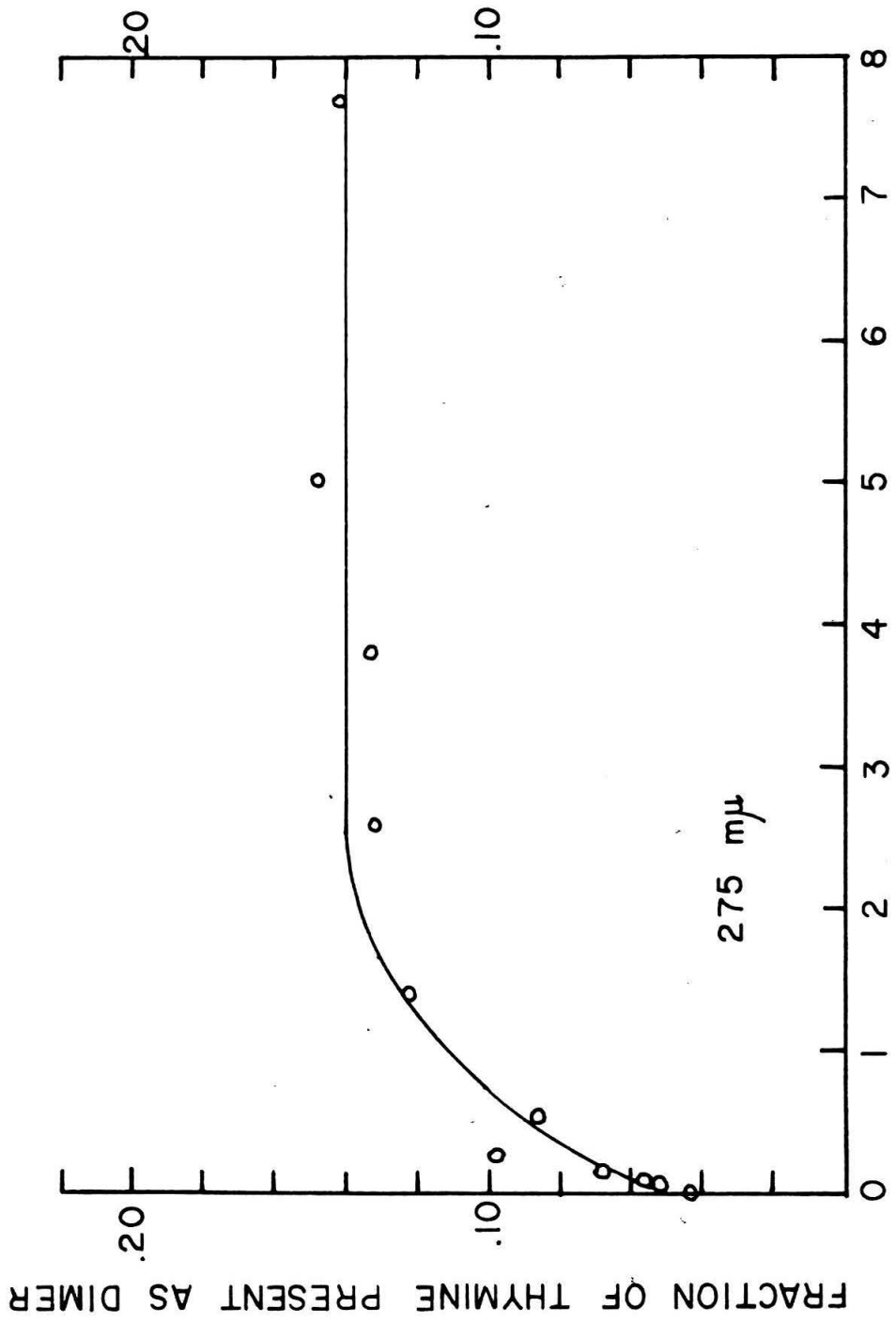

Fig. 5. Fraction of thymine present as dimer plotted against dose at 275 mμ for a sample which had first been given 10^6 ergs/mm^2 of 275 mμ light (fig. 3) and then 5×10^5 ergs/mm^2 of 250 mμ light (fig. 4).

Calculation of quantum yields for the dimerization. The probability that a given ultraviolet dose will cause one thymine molecule to become half of a thymine dimer molecule may be calculated from the initial slopes of the curves shown in figs. 1, 2 and 3. Knowing the extinction coefficients per mole of phosphorus for DNA (Table I), it is possible to calculate the quantum yields for the dimerization (Table II). The

Table I

Extinction coefficients for DNA per mole of phosphorus and for thymine dimer

$\lambda(m\mu)$	DNA* ϵ/P	Thymine dimer+ ϵ
235	3000	1570
254	6400	285
275	4500	14.8

*Courtesy Mr. R. Jensen

+Courtesy Dr. H. Johns

Table II

Quantum yield for dimerization

$\lambda(m\mu)$	Q
235	.009
254	.012
275	.016

Q = Fraction of thymine dimerized per quantum absorbed per nucleotide.

quantum yields increase slightly with increasing wavelength. The data are not quite accurate enough for one to be confident of this trend; in any case, it is clear that the quantum yields are not strongly dependent upon wavelength.

Ideal statistics of dimer formation in a polynucleotide chain. Before further analyzing dimer formation, it is necessary to present a model for thymine dimerization in DNA and to explore some of its predictions. This model is as follows: Photodimerization can take place between any two thymines which are adjacent to each other along a polynucleotide chain. The probability that an incident quantum will cause a photodimerization of an adjacent pair of thymines, X_m, is a constant independent of ultraviolet dose and independent of the identity of the surrounding nucleotides. Conversely, the probability that an incident quantum will cause a photodimer to revert, X_d, is also a constant independent of dose and the identity of surrounding nucleotides.

In order to calculate the position of the photosteady state in terms of X_m and X_d, it is necessary to make a further assumption about the distribution of thymine nucleotides in the polynucleotide chain. Suppose that we start at one end of the chain and proceed to the other, noting which of the four nucleotides immediately follows each thymine nucleotide. The probability, a, that a thymine nucleotide follows a thymine nucleotide is defined as the thymine-thymine nearest neighbor frequency. Moreover, the thymine-thymine nearest neighbors will occur

in sequences of two or more consecutive thymines, terminated at each end by a nucleotide different from thymine. The task at hand is to first express, in terms of X_m and X_d, the fraction of thymine dimerized at the photosteady state in a sequence of $\underline{n}$ consecutive thymines. Then, a random distribution of the thymine-thymine nearest neighbors in the polynucleotide chain will be assumed and the contribution made by each sequence of consecutive thymines to the position of the photosteady state will be assessed.

Let a sequence of $\underline{n}$ consecutive thymines be represented by a succession of $\underline{n}$ dots:

. . . . . .

Let a state in which two of these thymines are dimerized be represented by drawing a line between the dots representing the participating thymines, thereby converting two of the dots into one dash:

. . . . . — . . . . . .

If X_m is the probability of two dots going to a dash, and X_d the probability for the reverse process, then, at the photosteady state, the ratio of the concentration of any one state with one dash to the concentration of the state with no dashes is $(X_m/X_d) \equiv K$. Similarly, the ratio of the concentration of any one state with $\underline{p}$ dimers to the concentration of the state with no dimers is K^p. In general, there are many different states containing any particular number, $\underline{p}$, of dimers. The number of different states containing $\underline{p}$ dimers is just the number of

different ways of permuting p dashes and n - 2p dots, or

$$\frac{(n - p)!}{p!\,(n - 2p)!}$$

The system can be normalized by putting the sum of the relative concentrations of all the states equal to unity:

$$\sum_{p=0}^{p=n/2} \frac{(n - p)!}{p!\,(n - 2p)!} K^p = 1$$

The fraction of thymine present as dimer in a state containing p dimers is $2p/n$. Therefore, any one state containing p dimers contributes a fraction of

$$\frac{\frac{2p}{n} K^p}{\sum_{p=0}^{p=n/2} \frac{(n - p)!}{p!\,(n - 2p)!} K^p}$$

to the total amount of thymine present as dimer. The fraction of thymine present as dimer in a sequence of n consecutive thymines is then given by

$$f_n = \frac{\sum_{p=0}^{p=n/2} \frac{2p}{n} \frac{(n - p)!}{p!\,(n - 2p)!} K^p}{\sum_{p=0}^{p=n/2} \frac{(n - p)!}{p!\,(n - 2p)!} K^p} \qquad (1a)$$

It is possible to sum the series in equation la (67) to give

$$f_n = \frac{4K}{(1+4K)} \quad \frac{(\sqrt{1+4K}-1/n)(\sqrt{1+4K}+1)^n+(-1)^n(\sqrt{1+4K}+1/n)(\sqrt{1+4K}-1)^n}{(\sqrt{1+4K}+1)^{n+1} + (-1)^n (\sqrt{1+4K}-1)^{n+1}} \qquad (1b)$$

A random distribution of the thymine-thymine nearest neighbors in the polynucleotide chain will now be assumed and an expression for the fraction of thymine occurring in chains of $\underline{n}$ consecutive thymines will be derived: Arbitrarily choose a particular thymine in the polynucleotide chain. If the thymine-thymine nearest neighbor frequency is $\underline{a}$, the probability that this thymine will have something different from thymine on both sides of it is $(1 - a)^2$. The probability that it will have a thymine followed by non-thymine on one particular side of it and non-thymine on the other side is $a(1 - a)^2$. The probability that it will have a thymine on either the one side or the other of it, followed by terminating non-thymines will be $2a(1 - a)^2$. In general, the probability that the arbitrarily chosen thymine will have a particular number of thymines on one side of it and a particular number of thymines on the other side of it is $a^{n-1}(1 - a)^2$, where $\underline{n}$ is the number of thymines in the sequence. Since there are $\underline{n}$ ways that the thymine neighbors can be distributed between one side of the arbitrarily chosen thymine and the other, the probability that any thymine is a member of a sequence of $\underline{n}$ thymines is

$$na^{n-1}(1 - a)^2$$

The fraction of the total thymine present as dimer is then

$$F = \sum_{n=2}^{\infty} n a^{n-1} (1 - a)^2 f_n \qquad (2)$$

where f_n is given by equation 1a or 1b.

In E. coli DNA, the thymine-thymine nearest neighbor frequency is 0.30 (68). Using a=0.30 and equation 2, the fraction of thymine present as dimer has been plotted against K in fig. 6 for the case of a random distribution of thymine-thymine nearest neighbors. Of course, the distribution of thymine-thymine nearest neighbors along the chain need not be random. Using a = 0.30, the fraction of thymine present as dimer has been plotted against K in fig. 6 for the following cases: (1) All thymines occur in runs of 1 and 2 consecutive thymines (n = 2). (2) All thymines occur in runs of 1 and 3 consecutive thymines (n = 3). (3) All thymines are either isolated or occur in infinite chains of consecutive thymines (n = ∞).

Comparison of the observed photosteady states with those predicted by the model. In order to compare the photosteady state observed at a given wavelength with that predicted by the model, it is necessary to estimate the K characteristic of any given wavelength. To do this, the rate of the forward reaction is calculated from the initial rate of dimerization at the particular wavelength in question in the following way: The quantity directly measured is the fraction of total thymines dimerized per unit dose; the quantity desired is the fraction of thymine-

Legend for Fig. 6:

The solid lines signify the fraction of thymine present as dimer at the photosteady state (for $\underline{K}$ = 0.02 to $\underline{K}$ = 60) for various modifications of the model:

n = 2	Thymine-thymine nearest neighbor frequency 0.3; all thymines occur in runs of 1 and 2 consecutive thymines.
n = 3	Thymine-thymine nearest neighbor frequency 0.3; all thymines occur in runs of 1 and 3 consecutive thymines.
n = ∞	Thymine-thymine nearest neighbor frequency 0.3; all thymine-thymine nearest neighbors occur in long chains of thymines.
random	Thymine-thymine nearest neighbor frequency 0.3; distribution of thymine-thymine nearest neighbors is random along the polynucleotide chain.

The observed photostationary states for 235 mμ, 254 mμ, and 275 mμ are plotted against the $\underline{K}$ values for these three wavelengths. The $\underline{K}$ values were calculated from the initial measured rates of dimerization, the extinction coefficients for thymine dimer, and an assumed quantum yield of 0.6 for the back reaction, as explained in the text.

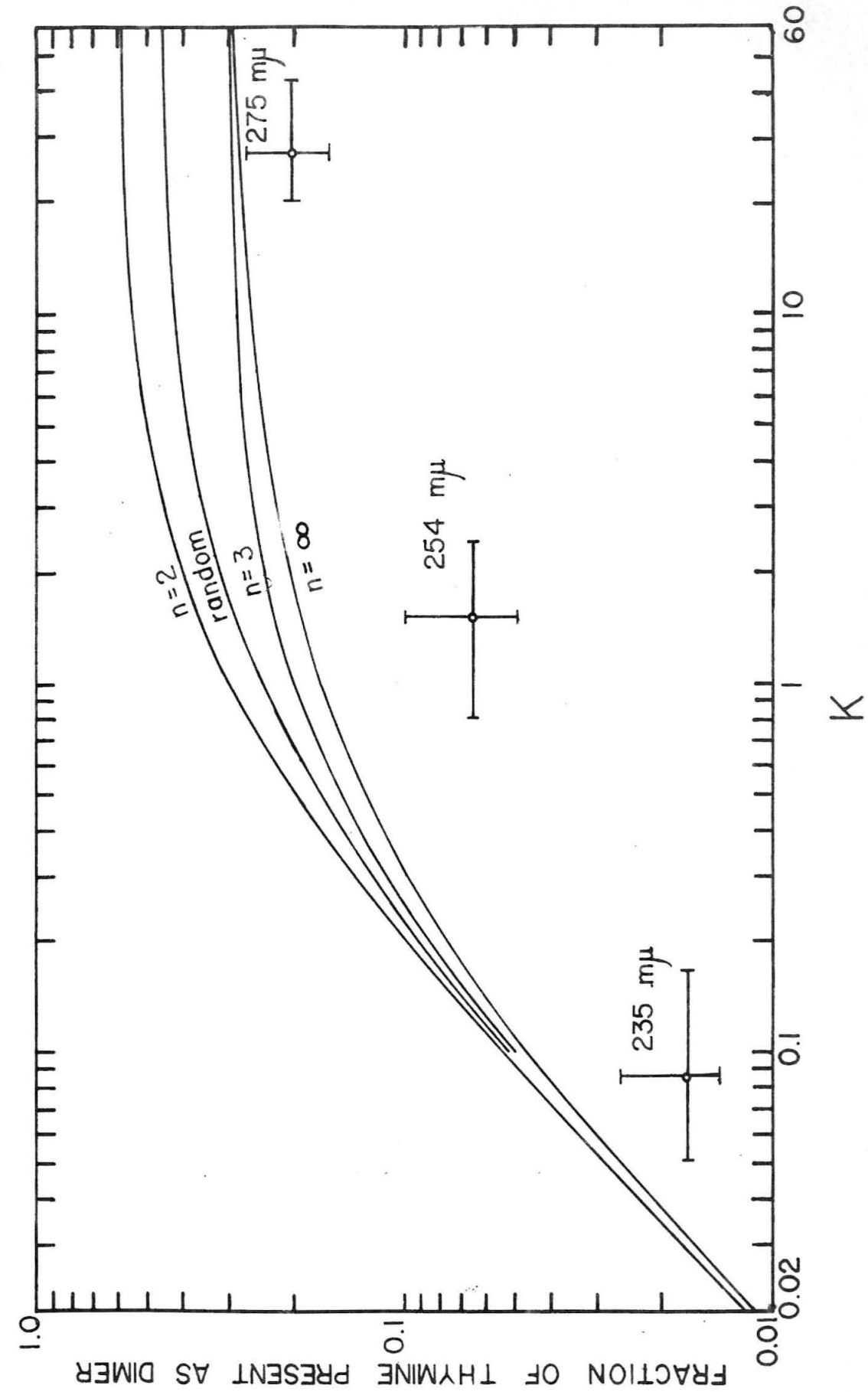
FRACTION OF THYMINE PRESENT AS DIMER
1.0
0.1
0.01
0.02
0.1
1
10
60
K
n=2
random
n=3
n=∞
275 mμ
254 mμ
235 mμ

Fig. 6.

thymine nearest neighbor pairs dimerized per unit dose. Taking the nearest neighbor frequency as 0.3, the fraction of nearest neighbor pairs dimerized per unit dose is 1/0.3 of the fraction of total thymines dimerized per unit dose. The rate of the reverse reaction is calculated by multiplying the extinction coefficients of thymine dimer in aqueous solution (Table I) by 0.6, the quantum yield for reversion of thymine dimer in aqueous solution (46,47). The photosteady state constants, $\underline{K}$, calculated in this manner are plotted in fig. 6 against the observed fraction of thymine dimer, together with an estimate of the uncertainty in the given values. The experimental uncertainty in $\underline{K}$ lies partly in the uncertainty in measuring the initial slope of the forward reaction and in a systematic error of $\pm$10% in measuring the fraction of thymine dimer. The uncertainty in the effective wavelength of irradiation (no more than 3mμ as discussed in the experimental section) introduces an uncertainty of up to a factor of 1.5 in the calculated $\underline{K}$. The uncertainty in the photosteady state lies in the systematic error of $\pm$10% in measuring the fraction of thymine dimer and the indication that the fraction of thymine dimer in the photosteady state slowly decreases, most clearly shown by comparing fig. 3 to fig. 5.

From fig. 6 it is clear that the fraction of thymine dimer observed at any given wavelength is significantly lower than that which was predicted. This anomaly is explained if either (1) the $\underline{K}$ calculated for a given wavelength is wrong or (2) the model does not accurately describe the system. If the $\underline{K}$ calculated for the system is wrong, then the

discrepancy might lie in the value assumed for the back reaction. (The rate of the forward reaction was directly determined from experimental data.) In order to make the observed values lie in the vicinity of the experimental curves one must multiply the cross-section for the reverse reaction, X_d, by about 2.5 at 235 mμ and by 10 or even more at 254 mμ and 275 mμ. There is one direct measure of the rate of the back reaction in DNA, namely the initial rate of dimer reversion found upon irradiating DNA containing 20% thymine dimer with 250 mμ ultraviolet light. The initial slope for dimer reversion (fig. 4) gives a quantum yield of 0.9, i.e., a cross-section for the reverse reaction which is 1.5 times that assumed in calculating K. In spite of the uncertainty in measuring the slope, the observed cross-section for the back reaction seems to be significantly less than the value one must assume in order to place the photosteady states observed at 250 mμ and 275 mμ in the vicinity of the theoretical curve. On the other hand, it is quite conceivable that the discrepancy at 235 mμ is due to using too low a cross-section for the rate of the back reaction.

It thus seems quite likely that the failure of the experimental points to fit the expected curves is due in large part to inadequacies in the model. Possibly K is not the same for all thymine-thymine nearest neighbor pairs. Possibly the rate of the forward reaction is dependent upon the fraction of thymine dimerized and/or other ultraviolet damages to the DNA in such a way as to make the initial K greater than

the K characteristic of the photosteady state.

It should be noted that Wacker (42) obtained a somewhat higher 254 mμ photostationary state than that reported here (9.7% of the thymine present as dimer instead of 6.5%). Although this result may be due to a difference in effective wavelength of irradiation, it also may result from the fact that I purified thymine dimer to a greater extent than Wacker. In order to understand the situation more fully, both thymine and thymine dimer should be highly purified so that one does not have the fear that the radioactivity he measures is largely due to some degradation product of thymine. Then one would be able to accurately estimate the quantum yield for irreversible destruction of thymine and calculate its effect on the position of the photosteady state. One could then evaluate the possibilities mentioned in the preceding paragraph.

In spite of these uncertainties, it is clear that the position of the photosteady state in DNA is strongly dependent upon the wavelength of light used for irradiation. Moreover, the fraction of thymine present as dimer at the 275 mμ photosteady state is much less than what was observed by Johns et al. (46) for thymine dinucleotide and the change of the fraction of total thymine present as dimer at the photosteady state with wavelength is much less marked than was observed for thymine dinucleotide. These results strongly indicate that a large fraction of the thymine in DNA either cannot undergo dimerization at all, or else dimerizes at a very slow rate.

VI

DISAPPEARANCE OF THYMINE PHOTODIMER IN ULTRAVIOLET IRRADIATED DNA UPON TREATMENT WITH A PHOTOREACTIVATING ENZYME FROM BAKER'S YEAST[a]

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A dimer of thymine is formed by ultraviolet irradiation of frozen aqueous solutions of thymine (Beukers and Berends, 1960 and 1961, Wang, 1961, and Wulff and Fraenkel, 1961).

Thymine dimer has been isolated by hydrolysis of ultraviolet irradiated DNA (Beukers, Ijlstra and Berends, 1960 and Wacker, Dellweg and Weinblum, 1960). This suggests the interesting hypothesis that formation of thymine dimer is the, or one of the, significant chemical events in ultraviolet damage of microorganisms. Furthermore, the discovery that short wavelength ultraviolet irradiation of dilute aqueous solutions of thymine dimer causes reconversion to thymine (Beukers, Ijlstra and Berends, 1959) leads to the speculation that "photoreactivation" of 254 mμ ultraviolet damage to living microorganisms by light of

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[a] The following is a verbatim copy of a manuscript to be published in Biochemical and Biophysical Research Communications.

wavelengths centering around 370 mμ might be due to a similar reconversion of thymine dimer to thymine. This hypothesis is supported by the present work in which it is shown that thymine dimer formed in irradiated DNA in vitro can be eliminated by illuminating the DNA in the presence of a photoreactivating enzyme from baker's yeast which repairs ultraviolet damage to bacterial transforming DNA (Rupert, 1960). (It may be recalled that irradiation of aqueous solutions of thymine dimer with light around 3700 Å in the absence of enzyme does not cause reconversion to thymine (Wang, 1960).)

Preparation and irradiation of H^3-thymine DNA. H^3-thymine DNA (1.2 μc/μg obtained by growing E. coli 15 $A^-T^-U^-$ in a medium containing methyl-H^3 thymidine) was purified by cesium chloride centrifugation (Marmur, 1961) and exposed to 3100 ergs/mm^2 of 254 mμ ultraviolet light in an apparatus described by Johns et al. (in press).

Treatment of H^3-thymine DNA with an enzyme from baker's yeast. Incubation mixtures, containing 2.5 μg/ml DNA and 1250 μg/ml of a partially purified (ammonium sulfate) preparation of yeast photoreactivating enzyme were warmed to 37° for 30 minutes either in the dark or illuminated with 2000 μ watts/cm^2 of 340-400 mμ light from suitably filtered "blacklight" fluorescent bulbs. On the basis of previous experience with transforming DNA, this would be expected to give maximum repair of the biologically significant damage. The mixtures were then deproteinized with 1/3 volume 6 M NaCl and chloroform-octanol.

Heat inactivated enzyme was prepared by warming a 2500 μg/ml enzyme solution to 65°C for 10 minutes. Photoreactivating enzyme is reduced to 5% activity in 2 minutes at 65°C (Rupert, 1961 and in press).

Analysis for thymine and thymine dimer. DNA was subjected to formic acid hydrolysis (.03 to .3 μg of DNA in 25 μl of formic acid) in evacuated tubes at 175°C for 30 minutes (Wyatt and Cohen, 1953). The yield of thymine dimer from irradiated DNA was independent of hydrolysis time over a range of 15 to 60 minutes at 175°C.

DNA hydrolysates, to which were added 10 μg carrier thymine dimer (prepared from u.v. irradiated frozen thymine solutions) and 10 μg carrier thymine, were paper chromatographed in isopropanol : conc HCl : water (68 : 15.5 : 16.5). Thymine dimer was further purified by paper chromatography in saturated ammonium sulfate : 1 N sodium acetate : isopropanol (40 : 9 : 1) (Wacker, 1960).

Tritium was counted in a liquid scintillation spectrometer, using the dioxane-water (15 ml : 1 ml) system of Butler (1961). The paper and salts from chromatography did not interfere with counting. The thymine activity constituted more than 98% of the total radioactivity in unirradiated DNA hydrolysates. The recovery of carrier thymine dimer (considerably less than 100%) was assayed spectrophotometrically after u.v. induced reconversion of thymine. (This estimation procedure was standardized with 10 μg carrier thymine dimer.)

Results and Discussion

In the second and third columns of Table I are tabulated the net radioactivities observed for thymine and thymine dimer in the various hydrolysates. In the fourth column the recovery of carrier thymine dimer is tabulated and in the fifth column the appropriate correction is made for the loss of H^3-thymine dimer upon chromatography. The fraction of thymine present as dimer is shown in the last column.

Table I

Sample	Thymine cpm	Observed Dimer cpm	% Dimer Recovery	Corrected Dimer cpm	Fraction of Thymine Present as Dimer
Unirradiated DNA	48884	8	91	-	-
	49671	6	66	-	-
u.v.'d DNA	50199	447	84	533	.011
	41971	418	86	483	.011
u.v.'d DNA + Enzyme in Dark	14124	81	54	151	.011
	15439	97	64	150	.010
u.v.'d DNA + Heated Enzyme in Light	10676	107	91	118	.011
	8109	84	88	95	.012
u.v.'d DNA + Enzyme in Light	9307	4	91	4	<.001
	8497	0	91	0	<.001

Results of duplicate hydrolyses and analyses of a single sample are listed. Columns explained in text.

The important result is that, whereas samples of irradiated DNA incubated with photoreactivating enzyme in the dark and samples incubated with heat inactivated enzyme in the light both show the same amount of thymine dimer as is present in the untreated irradiated DNA, incubation of irradiated DNA with enzyme plus light destroys over 90 percent of the dimer present.

A similar result was obtained independently by Wacker (1961), using a crude yeast extract which would be expected to contain photoreactivating enzyme. Both findings permit the interpretation that the enzyme causes the dimer in DNA to disappear upon incubation with light, presumably by converting it back to thymine.

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REFERENCES

R. Beukers and W. Berends, Biochem. Biophys. Acta, 41, 550 (1960).

R. Beukers and W. Berends, Biochem. Biophys. Acta, 49, 181 (1961).

R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 78, 883 (1959).

R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 79, 101 (1960).

F. E. Butler, Anal. Chem., 33, 409 (1961).

H. Johns, S. Rappaport, and M. Delbrück, in press.

J. Marmur, J. Mol. Biol., 3, 208 (1961).

C. S. Rupert, J. Gen. Physiol., 43, 573 (1960).

C. S. Rupert, J. Cell & Comp. Physiol., 58, Suppl. 1, 57 (1961).

C. S. Rupert, J. Gen. Physiol., in press.

A. Wacker, H. Dellweg and D. Weinblum, Naturwissenschaften, 47, 477 (1960).

A. Wacker, J. Chimie Physique, 58, 1041 (1961).

S. Y. Wang, Nature, 188, 846 (1960).

S. Y. Wang, Nature, 190, 690 (1961).

D. L. Wulff and G. Fraenkel, Biochem. Biophys. Acta, 51, 332 (1961).

G. R. Wyatt and S. S. Cohen, Biochem. J., 55, 774 (1953).

Addendum to the foregoing manuscript

Dr. Rupert has pointed out that the mechanism by which thymine dimer disappears upon incubation with the photoreactivating enzyme preparation could be quite different from the mechanism by which ultraviolet damage to the biological activity of transforming DNA is repaired. In a recent letter to me he stated:

> What we now know is that a heat-labile entity in our very impure enzyme preparation will act in the light to change thymine dimer in DNA to something else. We would like to think that this entity is the same as the photoreactivating enzyme which repairs UV damage in DNA. Simultaneous assay of dimerization and UV damage in the same DNA samples furnishes the means for testing this point. One should (1) Measure the fraction of dimer repaired and the fraction of UV damage repaired after partial photoreactivation of irradiated H^3 DNA, to determine if these two numbers correspond within the accuracy permitted by the data. This should be done (a) With enzyme of the present purity. (b) Enzyme of the highest purity possible. (c) Enzyme partly inactivated by heat or heavy metals. (d) With the reacting system illuminated at several different wavelengths showing different effectiveness for DNA repair.

REFERENCES

1. A. Rörsch, R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 77, 423 (1958).

2. R. Beukers, J. Ylstra and W. Berends, Rec. Trav. Chim., 77, 729 (1958).

3. R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 78, 247 (1959).

4. R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 78, 879 (1959).

5. R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 78, 883 (1959).

6. R. Beukers, J. Ijlstra and W. Berends, Rec. Trav. Chim., 79, 101 (1960).

7. R. Beukers and W. Berends, Biochim. Biophys. Acta, 38, 573 (1960).

8. R. Beukers and W. Berends, Biochim. Biophys. Acta, 41, 550 (1960)

9. R. Beukers and W. Berends, Biochim. Biophys. Acta, 49, 181 (1961).

10. W. Berends, J. Chim. Physique, 58, 1034 (1961).

11. D. Shugar in Chargoff and Davidson, The Nucleic Acids. Vol. III, Academic Press, pp. 39-103 (1960).

12. A. Canzanelli, R. Guild and D. Rapport, Am. J. Physiol., 167, 364 (1951).

13. M. Errera, Biochim. Biophys. Acta, 8, 30 (1952).

14. R. L. Sinsheimer and R. Hastings, Science, 110, 525 (1949).

15. R. L. Sinsheimer, Rad. Research, 1, 505 (1954).

16. S. Y. Wang, M. Apicella and B. R. Stone, J. Am. Chem. Soc., 78, 4180 (1956).

17. A. M. Moore and C. H. Thomson, Can. J. Chem., 35, 163 (1957).

18. A. M. Moore, Can. J. Chem., 36, 281 (1958).

19. S. Y. Wang, J. Am. Chem. Soc., 80, 6196 (1958).

20. A. M. Moore and C. H. Thomson, International Conf. on Radiobiology 4th Cambridge 1955, 75 (1955).

21. D. Shugar and K. L. Wierzchowski, Postepy Biochem., 4, suppl., 243 (1958).

22. A. M. Moore, Can. J. Chem., 37, 1281 (1959).

23. S. Y. Wang, Nature, 190, 690 (1961).

24. S. Y. Wang, Nature, 184, B. A. 59 (1959).

25. K. L. Wierzchowski and D. Shugar, Acta Biochim. Polonica, 7, 63 (1960).

26. R. L. Sinsheimer, Rad. Research, 6, 121 (1957).

27. K. L. Wierzchowski and D. Shugar, Biochim. Biophys. Acta, 25, 355 (1957).

28. K. L. Wierzchowski and D. Shugar, Acta Biochimica Polonica, 8, 219 (1961).

29. S. Y. Wang, Nature, 184, 184 (1959).

30. G. Hammond, private communication.

31. S. Y. Wang, private communication.

32. D. L. Wulff and G. Fraenkel, Biochim. Biophys. Acta, 52, 332 (1961).

33. D. L. Wulff, unpublished results.

34. R. F. Stewart, private communication.

35. K. Hoogsteen, Acta Cryst., 12, 822 (1959).

36. K. Hoogsteen, private communication.

37. S. Y. Wang, private communication.

38. R. Gerdil, Acta Cryst., 14, 333 (1961).

39. D. L. Wulff, unpublished observations.

40. A. Wacker, L. Träger and D. Weinblum, Angew. Chem., 73, 65 (1961).

41. A. Wacker, D. Weinblum, L. Träger and Z. H. Moustafa, J. Mol. Biol., 3, 790 (1961).

42. A. Wacker, J. Chim. Physique, 58, 1041 (1961).

43. A. Smietanowska and D. Shugar, Bulletin de l'Academie Polonaise des Sciences (Serie des Sciences Biologiques), 9, 375 (1961).

44. A. Wacker, H. Dellweg and E. Lodemann, Angew. Chem., 73, 64 (1961).

45. K. Smith, Biochem. Biophys. Res. Comm., 6, 458 (1962).

46. H. Johns, S. Rappaport and M. Delbrück, in press.

47. R. Setlow, Biochem. Biophys. Acta, 49, 237 (1961).

48. H. Moroson and P. Alexander, Rad. Research, 14, 29 (1961).

49. D. Shugar and K. L. Wierzchowski, J. Poly. Sci., 31, 269 (1958).

50. S. Y. Wang, Nature, 188, 844 (1960).

51. A. Wacker, H. Dellweg and D. Weinblum, Naturwissenschaften, 47, 477 (1960).

52. L. Grossman, D. Stollar and K. Herrington, J. Chim. Physique, 58, 1078 (1961).

53. J. Marmur, W. F. Anderson, L. Matthews, K. Berns, E. Gajewska, D. Lane and P. Doty, J. Cellular Comp. Physiol., 58, Suppl. 1, 33 (1961).

54. J. Marmur and L. Grossman, Proc. Nat. Acad. Sci., 47, 778 (1961).

55. R. Setlow and B. Doyle, Biochem. Biophys. Acta, 15, 117 (1954).

56. J. Baranowska and D. Shugar, Acta Biochimica Polonica, 7, 505 (1960).

57. F. Stahl in Burnett and Stanley, The Viruses. Vol. II, pp. 353-384 (1959). Academic Press (New York).

58. J. Jagger, Bacteriol. Rev., 22, 99 (1958).

59. C. S. Rupert, J. Gen. Physiol., 43, 573 (1960).

60. C. S. Rupert, J. Cell. & Comp. Physiol., 58, Suppl. 1, 57 (1961).

61. C. S. Rupert, J. Gen. Physiol., in press.

62. O. Maaløe and P. Hanawalt, J. Mol. Biol., 3, 144 (1961).

63. J. Marmur, J. Mol. Biol., 3, 208 (1961).

64. G. R. Wyatt and S. S. Cohen, Biochem. J., 55, 774 (1953).

65. F. E. Butler, Anal. Chem., 33, 409 (1961).

66. C. S. Rupert, private communication.

67. W. Huber, private communication.

68. J. Josse, A. Kaiser and A. Kornberg, J. Biol. Chem., 236, 864 (1961).

PROPOSITION #1

It is desirable to know if thymine dimer formed in u.v. irradiated DNA constitutes a lethal ultraviolet damage to microorganisms. It is proposed to u.v. inactivate the bacteriophage T2 to about 1 lethal hit per phage (37% survival) and to analyze for the fraction of thymine present as thymine photodimer, using phage labeled with H^3-thymine. If this constitutes on the order of one or more thymine dimers per phage, then thymine dimer may indeed be a primary cause of phage inactivation. By allowing the irradiated phage (labeled with H^3 DNA) to go through an infection cycle with its host (in nonradioactive medium) and analyzing for the fraction of H^3-thymine present as dimer in the progeny, one measures to what extent thymine dimer is a lethal photochemical alteration of bacteriophage.

In the case that no thymine dimers are found in the progeny, one would like to eliminate the possibility that this was merely because the thymine dimers had been chemically altered. To demonstrate that this has not happened, one should show that thymine dimer <u>is</u> transferred to progeny if the bacterium is coninfected with a high multiplicity of non-u.v.'d phage. (Hershey and Burgi (1) found that lethal u.v. damages in parental phage are transferred to progeny under these conditions.)

In detail, one prepares T2 labeled with 10 c/m$\underline{M}$ of thymine-H^3 by the method of Cairns (3), obtaining $\sim 6 \times 10^9$ phage/μg thymine. One irradiates to $\sim$37% survival ($\sim$ 55 ergs/mm^2) and analyzes one aliquot for thymine dimer. (If one thymine dimer is formed per phage, then the fraction of thymine dimerized is 2×10^{-5}. One needs $\sim$ 100 cpm to measure this with liquid scintillation counting or $\sim 4 \times 10^{-7}$ mc of H^3-thymine dimer. This means that one must analyze $\sim 2 \times 10^{-2}$ mc of H^3-phage or 10^{10} phage.)

To infect, one adds $\sim 2 \times 10^{10}$ H^3-phage to $\sim 6 \times 10^{10}$ bacteria in $\sim$400 ml. One induces lysis after 50 phage per infective center are formed. About 50% of the parental phage DNA will be transferred to the progeny (1). One then analyzes the $\sim 10^{12}$ progeny phage (containing $\sim$ 200 μg DNA) for thymine and thymine dimer. If thymine dimer is not transferred to progeny in single infection but is transferred to progeny in multiple infection, then the fraction of parental thymine present as dimer in the progeny will be $\sim$ 0.3 of the fraction of thymine present as dimer in the parents. If more than this amount is transferred to progeny, then not all thymine dimers are lethal in single infection. If less than this amount is transferred to progeny, that amount which has been transferred to progeny may or may not be entirely derived from bacteria infected with more than one phage.

To do the control experiment (second paragraph), one adds $\sim 2 \times 10^{10}$ H^3-phage and $\sim 3 \times 10^{11}$ cold phage to $\sim 6 \times 10^{10}$ bacteria in $\sim$400 ml. One proceeds as before, only this time one must analyze $\sim 3 \times 10^{12}$ progeny phage (containing $\sim$600 μg DNA).

REFERENCES

1. A. Hershey and E. Burgi, Cold Spring Harbor Symposia on Quantitative Biology, 21, 91 (1956).
2. For a general article on the u.v. inactivation of bacteriophage, see F. Stahl in Burnett and Stanley, The Viruses, Vol. II, pp. 353-384 (1959). Academic Press (New York).
3. J. Cairns, J. Mol. Biol., 3, 756 (1961).

PROPOSITION #2

There are several lines of evidence indicating that the common laboratory strains of E. coli can chemically repair ultraviolet damages (1). Two mutants of E. coli, strains Bs_1 and Bs_2, have been isolated which have high u.v. sensitivity (2, 3). The bacteriophage T1 has a ~6-fold increase in u.v. sensitivity when plated on strain Bs_1 (1). A method is proposed for selecting for mutant bacteria on which temperate phage have high u.v. sensitivity:

The bacterial population is infected with a lightly irradiated temperate phage at a rather high multiplicity (say λ on E. coli K12 at m=5 with a u.v. dose such that 90% of the non-mutated bacteria yield infective phage). Those bacteria which are u.v. resistant (i.e. have their repair mechanisms intact) will repair many of the ultraviolet damages in the absorbed phage. The phage will replicate, kill the bacterium, and progeny will be liberated. (Anti-λ serum must be added after absorption to keep these progeny phage from infecting the surviving bacteria.) Those bacteria which cannot repair u.v. damages will not repair the u.v. damages to the absorbed phage. As a result, the phage will remain inactivated and the host bacterium will live, inasmuch as u.v.-killed λ does not inactivate its host (4). A bacterium on which λ has a 5-fold increase in u.v. sensitivity will be concentrated by a factor of 5 to 10 in this way, as estimated from Fig. 4 of Kellenberger

and Weigle (5). After several cycles of infection with lightly irradiated phage, bacteria with high u.v. sensitivity will be concentrated with respect to the original culture.

The strains Bs_1 and Bs_2 were found among survivors of u.v. inactivated E. coli B. This kind of strain is apparently formed to about 1 or 2% when E. coli B is u.v. irradiated to 10^{-4} survival (3). If a similar strain is formed with about the same frequency from u.v. irradiated E. coli K12, then 3 cycles of the procedure outlined in the above paragraph would concentrate these mutants to greater than 50%.

It is also quite conceivable that this procedure, when used without prior u.v. inactivation, will select for different kinds of mutants than those obtained by the procedure of Hill.

REFERENCES

1. W. Sauerbier, in press.

2. R. Hill, Biochim. Biophys. Acta, 30, 636 (1958).

3. R. Hill and E. Simson, J. Gen. Microbiol., 24, 1 (1961).

4. J. Weigle, private communication.

5. G. Kellenberger and J. Weigle, Biochim. Biophys. Acta, 30, 112 (1958).

PROPOSITION #3

There is some interest in these laboratories in measuring the polarization of electronic transitions in thin crystals of purines and pyrimidines in order to better understand the electronic behavior of nucleic acids and their components. Since one wishes to relate the preferred direction of absorption of light in the crystal to the preferred direction of absorption of light in the molecules, only crystals of known crystal structure are useful. One limitation in this work is the limited number of crystals of known structure which are available.

It is proposed that information regarding the polarization of electronic absorption in the thymine analog 5-bromouracil (5-BU) (crystal structure unknown) may be obtained by "doping" calcium thymidylate crystals, whose crystal structure is known (1), with the calcium salt of 5-bromouracil deoxyriboside-5'-phosphate and making the reasonable assumption that the orientation of this 5-BU derivative in the unit cell is the same as the orientation of calcium thymidylate.

One most establish that the "doped" crystal has the same arrangement of calcium thymidylate molecules as the pure calcium thymidylate crystal. A reasonable criterion for this is that the unit cell dimensions in the doped crystal must be the same as in the pure crystal (2). The maximum amount of the 5-BU derivative one can thus incorporate into the calcium thymidylate structure will determine the spectral range over

which 5-BU transitions may be studied. Since 5-BU absorbs much more strongly than thymine in the region of 300 to 320 mμ, only a few percent 5-BU incorporation will be necessary in order to observe the absorption of light by the 5-BU moiety in this spectral region. It would even be possible to determine the transition moment for 5-BU at its absorption maximum, where the thymine moiety absorbs a comparable amount of light, if the crystal structure of the 5-BU derivative is isomorphous with the crystal structure of calcium thymidylate. (If the unit cell dimensions of the calcium salt of 5-bromouracil deoxyriboside-5'-phosphate and calcium thymidylate are the same, then these crystals are quite likely isomorphous (2).)

REFERENCES

1. K. N. Trueblood, P. Horn, and V. Luzzati, Acta Cryst., 14, 965 (1961).

2. K. Hoogsteen, private communication.

PROPOSITION #4

The u.v. survival probability of a temperate bacteriophage is higher if the host bacterium is first given a light dose of u.v. (1, 2). This so-called "u.v. reactivation" has been interpreted as due to genetic exchange between genetically homologous regions of the lightly u.v. irradiated host and the infecting u.v. irradiated phage, with the resultant formation of undamaged phage genomes. (Pre-irradiation of the host is supposed to make the phage survival probability higher by encouraging this crossover.) It is proposed to test this hypothesis by techniques of bacterial conjugation:

A lightly u.v. 'd, λ-resistant Hfr bacterium is conjugated with a non-u.v. 'd λ-sensitive F^- bacterium and the "u.v. reactivability" marker is mapped by stopping conjugation at various times and assaying for the u.v. survival of λ on the F^-. If the interpretation of Garen and Zinder is correct, the "u.v. reactivability" marker should coincide with the site of λ lysogenization. In the case of a u.v. reactivable phage different from λ, the "u.v. reactivable" marker should coincide with the site of lysogenization of that phage with the bacterial genome.

REFERENCES

1. A. Garen and N. Zinder, Virology, 1, 347 (1955).

2. G. Kellenberger and J. Weigle, Biochim. Biophys. Acta, 30, 112 (1958).

3. For a discussion of the E. coli K12 conjugation system, read Jacob and Wollman, Sexuality and the Genetics of Bacteria, Academic Press, New York (1961).

PROPOSITION #5

A procedure for estimating the stability of cytosine photohydrate in irradiated DNA is proposed: If an aqueous solution of cytosine labeled with H^3 on the 5-carbon atom is irradiated, the cytosine water adduct will have an H^3 atom and an H^1 atom attached to its 5-carbon atom (see page 4 of this thesis). If this adduct now reverts back to cytosine, a tritium atom will sometimes be eliminated from the 5-position instead of the proton. To estimate the stability of cytosine hydrate in irradiated DNA, one incubates the DNA at a given temperature and determines the rate of H^3 appearance in the water by quenching aliquots in dry ice and subliming the water away from the DNA. (Cytosine hydrate is not decomposed under these conditions (1).) In order to calculate the rate constant, k, one assumes first order kinetics for the elimination and that the relative efficiency of H^3 to H^1 elimination remains constant over the course of the reaction. It should be noted that one is actually measuring the stability of the photohydrate of cytosine-H^3 in DNA, which will be different from the stability of the photohydrate of cytosine-H^1 DNA, depending on the magnitude of the isotope effect involved.

Since the release of H^3 into the water may be quite inefficient, one must be able to prepare DNA containing cytosine-5-H^3 at high specific activity. This may be done by growing E. coli 15 $T^-A^-U^-$ on

uracil-H^3 (2) and isolating its DNA. Uracil-H^3 (3 c/m$\underline{M}$) may be purchased from New England Nuclear Corporation for $50/mc (3). I do not know how the H^3 is distributed between the 5 and 6 carbon atoms. It should be possible to make uracil-5-H^3 of extremely high activity by reducing 5-bromouracil with tritium gas in the presence of Pd-C (4). One must use a non-hydroxylic solvent to prevent exchange of the tritium gas with the solvent (3). Fully tritiated uracil-5-H^3 has a specific activity of $\sim$30 c/m$\underline{M}$. New England Nuclear Corporation will react 15 curies of tritium gas with any submitted compound for $200 (3). One might reasonably hope to obtain $\sim$ 1.5 c of $\sim$10 c/m$\underline{M}$ uracil-5-H^3 in this way. Using the growth conditions of Maaløe and Hanawalt (2), one might reasonably hope to obtain $\sim$100 mc of DNA-cytosine-5-H^3 of specific activity $\sim$10 mc/mg. One could analyze 1 mc aliquots for release of tritium into the water. If the non-u.v. induced rate of H^3-exchange with the water is negligible, one now has a very sensitive way of measuring u.v. induced release of tritium into the water:

Ultimately about 100 cpm of tritium (using liquid scintillation counting) must be released into the water following irradiation in order to obtain sufficiently accurate data. Assuming a 10% counting efficiency, this corresponds to 4×10^{-7} mc of tritium. If one irradiates to 1% of the cytosines hydrated and analyzes 1 mc aliquots, at least one out of every 25,000 hydrogens coming off the 5-carbon atom of the cytosine-5-H^3 photohydrate must be a tritium in order for one to be able to measure the stability of cytosine hydrate in u.v.-irradiated DNA.

REFERENCES

1. K. L. Wierzchowski and D. Shugar, Acta Biochim. Polonica, 8, 219 (1961).

2. O. Maaløe and P. Hanawalt, J. Mol. Biol., 3, 144 (1961).

3. New England Nuclear Corp., Catalog, 1961.

4. S. Y. Wang, J. Am. Chem. Soc., 80, 6196 (1958).